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Sexual compatibility and seed germination in Nolana species

Amy C. Douglas
University of New Hampshire, Durham

SEXUAL COMPATIBILITY AND SEED GERMINATION IN *NOLANA* SPECIES

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AMY C. DOUGLAS
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This thesis has been examined and approved.

Thesis Director, Dr. Rosanna Freyre,
Research Scientist, University of Florida

Dr. Paul R. Fisher,
Associate Professor in Environmental Horticulture, University of Florida

Dr. Thomas Davis,
Professor of Plant Biology/Genetics

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Date

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ABSTRACT

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Amy C. Douglas

University of New Hampshire, September, 2007

Nolana L.f. is a large, diverse genus in the Solanaceae endemic to coastal deserts of Peru and Chile. Large showy flowers and drought tolerance give *Nolana* great potential for breeding and cultivar development for the ornamental plant industry. As a precursor to breeding efforts, studies of floral development, sexual compatibility, and seed germination were conducted involving eight *Nolana* species (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata, N. rupicola*) cultivated at UNH.

Stigma receptivity and pollen viability were evaluated at a range of stages of flower development. Floral development keys were developed to provide visual reference correlating morphological appearance of buds/flowers at each developmental stage to stage durations and levels of stigma receptivity.

Artificial hybridizations (self- intra- and interspecific) were performed within and between each species. Species were generally self-incompatible. Intraspecific compatibility was high. Success of interspecific hybridization was analyzed based on fruit set, mericarps per fruit, mericarp size, and seed germination. Reduction in interspecific fertility was generally seen as lower fruiting success and smaller mericarp

size as compared to intraspecific hybridization. Estimated seed counts were made by x-ray analysis of mericarps revealing differences in seed set between crosses. Hybrid seed was germinated verifying compatibility of 22 unidirectional species pairs.

Causes and remedies for low seed germination rates were investigated with analysis of mericarp morphology, imbibition, and effect of chemical and environmental germination treatments. Scanning electron microscopy and imbibition studies ruled out presence of physical germination barriors. Gibberellic acid (1000 ppm) effectively increased germination in some species. Older mericarps (stored dry for two years) had higher germination than fresh mericarps. Mericarps of *N. aticoana* stored for seven weeks at 35°C and 75% RH showed higher germination than mericarps stored dry, or stored moist for 1-6 or 8-12 weeks. Germination of seed cultured when immature did not surpass that of mature seed. X-ray analysis confimed existence of at least one seed within most mericarps. Germination rates fall far below theoretical potentials based on x-ray seed counts. Findings suggest germination failure is likely due to physiological dormancy rather than low seed set.

CHAPTER I

FLORAL DEVELOPMENT IN EIGHT *NOLANA* SPECIES

Additional Index Words
stigma receptivity, pollen viability

Abstract

Nolana L.f. is a large, diverse genus in the Solanaceae endemic to the coastal deserts of Peru and Chile. Floral development studies were conducted as a precursor to breeding efforts and studies of sexual compatibility. Levels of stigma receptivity and/or pollen viability were evaluated at a range of stages of flower development in eight species (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata,* and *N. rupicola*). Species were found to be receptive to pollination over a wide range of developmental stages, including stages prior to anthesis. Pollen was found to remain viable throughout the open flower period and into senescence. Floral development keys were developed which provide a visual reference correlating morphological appearance of buds and flowers of each species at each developmental stage to durations of each stage and to levels of stigma receptivity at each stage.

Introduction

Nolana is the fifth largest genus in the Solanaceae, with 85 species currently described (Dillon et al., 2003). *Nolana* is endemic to the coastal desert in Peru and the Atacama Desert in northern-central Chile. The majority of species are found between 7°59' and 33°21' S latitude, at 50-600 meters altitude, and within a few kilometers of the

Pacific coast (Mesa, 1986; Dillon et al., 2003). Most species are found in fog-dependent isolated patches of vegetation called *lomas* formations, and flourish during El Niño years when the *lomas* experience high rainfall and humidity (Tago-Nakawaza and Dillon, 1999). The genus was previously considered to belong to a distinct family, Nolanaceae, due to its 5-carpelled gyonecium, but was recently included in Solanaceae based on chloroplast DNA analysis (Olmstead and Palmer, 1992).

The fruit in *Nolana*, the mericarp, is a unique, derived character in the Solanaceae (Knapp, 2002). Fruit morphology varies by species with fruit reportedly consisting of two to 30 highly sclerified mericarps, and mericarps being unilocellate or plurilocellate, reportedly containing one to seven individual seeds each (Tago-Nakazawa and Dillon, 1999). Each seed has an associated funicular plug which is displaced upon germination providing a canal through which the seedling exits the mericarp (Bondeson, 1986).

Nolana species range from herbaceous annuals to moderately woody perennial shrubs (Tago-Nakawaza and Dillon, 1999). Most species display showy flowers borne singly in leaf axils. Flowers are tubular-salverform to campanulate, infundibular, or rotate (Freyre et al., 2005). Flowers range in size (1 cm diameter to 8 cm diameter) and in color (blues, purples, pinks, and whites).

Though largely unexploited, there is great potential to use *Nolana* in breeding programs to develop new ornamental cultivars. Desirable traits such as the large showy flowers, compact growth habit, and drought tolerance can be found throughout the genus, but cannot be found all within a single species. In order to develop ideal ornamental cultivars, it is necessary to combine characteristics of multiple *Nolana* species through creation of interspecific hybrids.

A *Nolana* breeding program was initiated at the University of New Hampshire (UNH) in 2001 with the goal of developing new *Nolana* cultivars for the ornamental plant industry. Since the program's inception, UNH has built a germplasm collection consisting of 22 *Nolana* species. Through controlled interspecific hybridizations, researchers aim to develop hybrid individuals possessing unique combinations of desirable morphological and physiological traits. In addition, studies of sexual compatibility between *Nolana* species are being conducted because reports on this topic are limited (Saunders, 1934; Freyre et al., 2005).

A prerequisite for breeding and studies of sexual compatibility between plant species is an understanding of the species' floral development schedules. Studies of sexual compatibility rely on the use of receptive stigmas and viable pollen in compatibility tests. Using only stigmas and pollen that are at the appropriate developmental stage for maximum fertilization potential allows for increased confidence that failure of a hybridization to produce viable seed is truly caused by sexual incompatibility rather than incorrect timing. Even highly compatible hybridizations will not produce viable seed if the pollinated stigma is at a developmental stage in which it is not receptive to pollen or if the pollen is too immature or too old to germinate. The ability to identify stigmas and pollen of the appropriate developmental stage is therefore crucial to successful sexual compatibility studies.

An understanding of a species' floral development schedule is likewise critical to an efficient breeding program. A concern to breeding is the potential for contamination of controlled pollinations by foreign pollen carried by insects or other pollinators. It has been reported that stigmas of some members of Solanaceae such as *Capsicum annuum*

and *Cyphomandra endopogon* become receptive to pollination several days prior to anthesis (Aleemullah et al., 2000; Gracie, 1993). In species such as these, threat of insect pollination may be reduced by removing the corolla at bud stage and pollinating the newly exposed stigma with desired pollen. When this technique is used, it is critical to know at what stage the stigma becomes receptive and pollen dehisces to ensure buds of appropriate age are selected.

Studies of floral development schedules in *Nolana* have not previously been reported. The goal of the present study was to characterize the floral development schedules of selected *Nolana* species and to associate timing of major developmental events including corolla growth and expansion, pollen dehiscence, and timing of stigmatic receptivity with visually distinct developmental stages. With these data, researchers can identify appropriate flowers for use in hybridizations by visual inspection of the pool of available flowers. This study was conducted in four stages with the following objectives: 1) document the progression of floral development in *Nolana* species from an immature bud stage through senescence, recording daily growth measurements and timing of visually identifiable developmental events including corolla opening, pollen dehiscence, and senescence; 2) identify similarities in timing of stigma receptivity and pollen viability using manual pollination and chemical enzyme staining in two species with differing developmental schedules; 3) survey pollination success across the range of developmental stages in multiple *Nolana* species to verify that similarities identified in objective 2 are consistent across species and can be used to make generalizations about appropriate timing of manual pollinations; 4) construct floral development keys illustrating developmental stages and corresponding durations and

levels of pollination success in each studied *Nolana* species for use as visual reference in artificial hybridizations.

Materials and Methods

Plant material

Eight *Nolana* species were initially selected for use in these studies (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata*, and *N. rupicola*). Selected species display a range of morphological traits represented within *Nolana* and are endemic to a range of locations within the genus' natural habitat, including six locations in Peru and two locations in Chile (Table 1.1).

Depending on availability, plant material included three to five accessions for each of eight *Nolana* species, which were vegetatively propagated and grown to maturity. Detailed collection information for plant material is reported in Table 1.1. Herbarium vouchers for all accessions are housed at the Hodgdon Herbarium, University of New Hampshire, and at the Field Museum of Natural History, Chicago, IL.

Plant material was maintained in an insect exclusion, double-poly hoop house at the University of New Hampshire's Woodman Farm. Plants were grown in 25-cm pots with Sunshine LA4 aggregate mix (SunGro Horticulture Inc., Bellevue, WA). Fertilization was constant with a 20N-4.3P-16.7K fertilizer at a maximum 150 $mg \cdot L^{-1}N$. Average air temperature was recorded for each stage of the study with a HOBO temperature logger (H08-001-02, Onset Corp., Bourne, MA). Average daily air temperatures were 21.1°C and 25.8°C for the periods of December 23, 2004 through January 13, 2005 and July 7, 2005 through August 15, 2005 respectively.

Floral development tracking

Floral development was tracked on one plant each of three to five accessions from each of seven *Nolana* species between the dates of December 23, 2004 and January 13, 2005. *N. rupicola* was omitted from this stage of the study because of insufficient flowering. Plants were grown under natural daylength and light intensity. Five buds per plant were tagged with an identifying number at an early developmental stage in which the length of the bud's corolla was shorter than that of its calyx (Figure 1.1 column *a*). Each bud was observed daily between 8 am and 10 am, noting the relative length of the closed corolla to that of the calyx. Data collection was initiated when the tip of the corolla was even in length with the tips of the calyx (Figure 1.1 column *b*, the *Even* stage), or when the length of the corolla surpassed that of the calyx if the preceding stage occurred between daily observations. Each individual bud was measured daily throughout development until senescence using digital calipers (MarCal 16EX). The length of the corolla was measured from the point of attachment of the peduncle to the tip of the corolla.

As floral development progressed, timing of visually identifiable developmental events were noted. Timing of corolla opening and senescence were recorded in all species as well as timing of visual anther dehiscence. In some species, opening of the corolla was observed in two distinct phases (recorded separately) with the corolla first becoming loose and slightly open at the tips, followed by subsequent full expansion of the corolla (Figure 1.1 columns *d*, *e*). Similarly, in some species, a prolonged senescence process was observed (also recorded in two phases) with fading and weakening of the corolla tissue followed by collapse of corolla tissue indicating full senescence (Figure 1.1

columns *f, g*). Measurements of the depths and diameters of the fully expanded corollas were made in all species (Table 1.5). Corolla colors were documented according to R.H.S. Colour Charts (Royal Horticultural Society, 1995).

Average stage durations of species and accessions were rounded to the nearest whole number to remain consistent with 24 h observation intervals (Tables 1.2, 1.3). Analysis of variance was performed to compare the number of days at each stage of development between and within species. Differences in duration of stages indicated by ANOVA were analyzed using Tukey-HSD (Systat 10, SPSS Inc, 2000).

Stigma receptivity and pollen viability

Timing of stigma receptivity and pollen viability was investigated in one accession each of *N. humifusa* and *N. laxa* (Hu9-4 and La1-2, respectively) between the dates of July 7, 2005 and August 5, 2005. Stigma receptivity was also investigated in one accession of *N. adansonii* (Ad4-11) during this period.

Eighteen immature buds for each day of floral development from the *Even* bud stage through senescence were tagged on *N. humifusa* and on *N. laxa* (108 buds on Hu9-4 and 252 buds on La1-2) and three buds per day were tagged on *N. adansonii* (45 buds). Numbers of days of floral development were calculated based on data obtained through floral development tracking.

Procedures were performed daily between 8 am and 9 am. On each day of development starting on the day when the bud reached or surpassed *Even* stage, stigmas of three buds were pollinated with pollen from recently opened flowers of a highly compatible individual of the same species, as determined by pilot studies (Hu9-4 x Hu1-2; La1-2 x La1-4; Ad4-11 x Ad4-14). Additionally, three buds each of *N. humifusa* and

N. laxa were pollinated each day with pollen from a different species (Hu9-4 x *N. aticoana* A2; La1-2 x *N. plicata* P5). Success or failure of fruit set was recorded for each pollination. A final set of three stigmas per day of *N. humifusa* and *N. laxa* were tested for the presence of dehydrogenases as an indicator of receptivity, by treatment with 3-4.5 Dimethylthiazol-2-yl-2.5-diphenyl-tetrazolium bromide (MTT) as described (Dafni et al., in press). Stigmas were treated with 20 g/L MTT in 5% sucrose at 28°C. Location and intensity of the stain were observed by microscopy within 30 min of initiation of staining.

Additionally, pollen viability was measured in buds of *N. humifusa* and *N. laxa* representing each day of development starting on the day the bud reached or surpassed *Even* stage. All flowers to be pollinated were emasculated at the *Even* stage to avoid possible interference by self pollen. For each day of development, pollen from three buds of Hu9-4 and La1-2 was applied to mature stigmas of a highly compatible individual of the same species (Hu1-2 x Hu9-4; La1-4 x La1-2). Pollen from a second set of three buds from Hu9-4 and La1-2 was applied to mature stigmas of a highly compatible individual of a different species (*N. aticoana* A2 x Hu9-4; *N. plicata* P5 x La1-2). Success or failure of fruit set was recorded for each pollination. Pollen from a final set of three buds per day was tested for the presence of dehydrogenases by treatment with 3-4.5 Dimethylthiazol-2-yl-2.5-diphenyl-tetrazolium bromide (MTT) as described above for stigmas.

Pollination success at a range of developmental stages

Plant material consisted of one accession of each of five *Nolana* species (*N. aticoana*, *N. humifusa*, *N. ivaniana*, *N. plicata*, and *N. rupicola*). Data collection took place between August 4, 2005 and August 15, 2005.

Six visually distinct floral development stages were designated in each species. These stages are described in the results section, and include *Calyx*, *Even*, *Closed corolla*, *Partially open*, *Open flower*, and *Wilted*. On each plant, five buds or flowers per developmental stage were pollinated with pollen applied from freshly opened flowers of a highly compatible individual of the same species, as indentified by piolet studies. Exclusion of pollinators from the greenhouse and care to avoid movement of plants prevented self-pollination in dehiscent flowers. Success or failure of fruit formation was recorded for each pollination.

Construction of floral development keys

Data were compiled to create graphical representations of floral development in each studied species. Buds and flowers representative of each stage of floral development as designated and described in the results section were photographed for each species. Photographed stages include *Calyx*, *Even*, *Closed corolla*, *Partially open*, *Open*, *Wilted*, and *Senescent*. Data of pollination success at a range of developmental stages are included as bar graphs corresponding to each photograph. Graphs representing pollination success in *N. laxa* and *N. adansonii* were compiled using data collected in stigma receptivity studies. Pollination success data was not available for *N. elegans* due to insufficient flowering. Timelines above the photographs represent the average developmental stage observed on each day of floral development tracking. In all cases,

day zero begins at the *Calyx* stage and continues to the stage observed on day one of observations. Each numbered division represents the developmental stage observed on the corresponding day of observation.

Results and Discussion

Floral development tracking

Floral development tracking provides a visual timeline of flower development in each of the studied species. These data allow generalizations to be made about durations of developmental stages in each species, providing the necessary background for relating timing of stigma receptivity and pollen viability to visually identifiable stages.

Each *Nolana* species studied is unique in bud morphology. Therefore, buds of equivalent physiological ages could not be identified for use as a starting point for data collection. Instead, we designated a consistent starting point in the early stages of bud development which could be easily identified in each species by visual inspection. This was the point at which the tips of the immature closed corolla were even in length with the tips of the calyx. This point may not be physiologically comparable between species. Therefore, data may only be interpreted as durations between developmental events with this identified stage being a key reference point.

Despite the unique morphologies of the species, equivalent stages of development based on morphological appearance can be assigned to each species. These stages have been designated by visual appearance only and do not represent physiological stages. Based on the observational data collected in this study, the morphological development of *Nolana* flowers can be characterized into seven unique developmental stages:

- *Calyx:* Immature bud stage in which length of the corolla is less than that of the calyx. Depending on the species, the corolla may be enclosed within a sealed calyx or may be visible within an open-ended calyx. This stage includes all bud development occurring prior to the start of data collection.
- *Even:* The point at which the length of the corolla is even with that of the calyx. This is the point designated as the starting point of data collection.
- *Closed corolla:* The corolla remains tightly closed, but has exceeded the length of the calyx and is continuing to elongate.
- *Partially open:* The corolla has begun to loosen and its tips are slightly apart. This stage includes the time during which the corolla continues to unfold and expand.
- *Open flower:* Begins when the corolla has fully unfolded.
- *Wilted:* Flowers have lost their vibrant color and have taken on a washed-out appearance. Corolla tissue becomes limp and often reflexes.
- *Senescent:* Corolla tissue collapses and the flower loses its open shape. Corolla tissue may hang loosely from the calyx or may begin to dry and shrivel, and eventually drops.

Duration of key stages in each of the studied species is summarized in Table 1.2. Values represent the average number of days for which buds of each species were observed as being in a particular developmental stage. Not all designated stages were observed in each bud. In some cases, a developmental stage was reached and surpassed between daily observations. For example, buds of the species *N. humifusa* developed from the *Calyx* stage to the *Partially open* stage within one day, with the *Even* and

Closed corolla stages occurring between observations. This occurrence is represented by zeros in the *Even* column and in the *Closed corolla* column of Table 1.2. Likewise, the *Wilted* stage was not observed in *N. aticoana*, *N. humifusa* or *N. ivaniana*, with flowers progressing from *Open flower* to *Senescent* in one day.

Analysis of variance was used to determine whether differences exist between species in terms of developmental schedules. Duration of each stage of development was compared between species. Results indicate highly significant differences between species in duration of developmental stages ($p < 0.00$ in all cases). Species are not all alike in regards to duration of developmental stages, thus the genus cannot be generalized in this regard. Differences between species in regards to a single stage of development, *Closed corolla*, are indicated in Table 1.2. This stage was selected to illustrate differences based on its significance as the target stage for controlled hybridizations.

Differences in stage durations within species were minimal in most cases. Table 1.3 reports exceptions. Only cases with greater than one day difference in stage duration are considered. Differences were identified by ANOVA between accessions in *N. humifusa*, *N. ivaniana*, and *N. plicata* in duration of *Open flower* stage ($p=0.000$, $p=0.000$, $p=0.006$, respectively). Differences between accessions of *N. adansonii* were most pronounced, with significant differences identified in duration of *Closed corolla*, *Partially open*, and *Open flower* stages ($p=0.000$ in all cases).

Flowers of *N. adansonii* accessions Ad2-3 and Ad4-1 failed to open fully and senesced directly from *Partially open* or *Closed corolla* stages. This is represented by zeros in the *Open* column of accession Ad2-3 and in both the *Partially open* and *Open* columns of accession Ad4-1 in Table 1.3. This phenomenon was not observed in these

plants upon subsequent observation during summer months suggesting that failure of flower opening was environmentally induced, either due to lower light intensity, shorter daylength, or lower average daily temperatures in the winter months.

Each bud was examined by visual inspection for the presence of dehiscent pollen at the earliest stage possible without disruption of the corolla. In many buds it was possible to peer into the partially opened corolla to observe the anthers, while in others anthers were not visible until further expansion of the corolla. In all but two buds (one bud of *N. elegans* accession Ele1 and one bud of *N. aticoana* accession A2), pollen was dehiscent upon first inspection of the anthers. In the two exceptional cases, pollen was observed as being dehiscent at the following observation, one day after corolla opening. In all other cases, the timing of pollen dehiscence (whether it occurs prior to corolla opening or at the time of corolla opening) could not be determined by the methods used in this study. However, the presence of dehiscent pollen at the early stages of flower opening suggests that emasculation at an earlier stage of bud development is necessary to avoid the presence of self pollen on stigmas.

Daily measurements of bud growth are reported in Table 1.4. In species with a prolonged *Closed corolla* stage, these data may be used to estimate the age of a particular bud in terms of days from opening. With further investigation into timing of pollen dehiscence, these bud measurements may be used to estimate the appropriate timing for emasculation to prevent self pollination.

Measurements of corolla diameters, depths, and colors are reported in Table 1.5. Flowers ranged from shallowly to deeply infundibular (funnelform). Flowers ranged in size from 5.4 cm diameter and 3.3 cm depth in *N. rupicola* to 2.6 cm diameter and 1.3 cm

depth in *N. humifusa*. Colors ranged from bright blue in *N. elegans* and *N. rupicola*, to pale blue in *N. aticoana*, *N. humifusa*, and *N. plicata*, bright purple in *N. adansonii* and *N. laxa*, and pale purple in *N. ivaniana*. Conspicuous darker colored veins and bicolored throats were apparent in some species.

Stigma receptivity and pollen viability

Based on data collected in floral development tracking, two species were selected for studies of timing of stigma receptivity and pollen viability. *N. humifusa* and *N. laxa* were selected as representative species with extreme differences in duration of developmental schedules. Total average duration of observed developmental stages in *N. humifusa* and *N. laxa* lasted four days and 12 days, respectively. It was hypothesized that each of these species would exhibit a peak in stigma receptivity and in pollen viability which could be correlated to specific floral development stages. If these two species with extremely different developmental schedules exhibited similarities in this respect, it is likely that other *Nolana* species would also exhibit similar patterns of stigma receptivity and pollen viability. One accession of *N. adansonii* was also included in the study of stigma receptivity.

As opposed to the previous study, this experiment was performed during summer months in July and August. Temperature- and/or light-dependent differences in the duration of developmental stages were evident in this study as compared to data from the floral development tracking study. Complete development of *N. humifusa* (Hu9-4) from *Even* stage to *Senescence* took an average of four days when measured previously during winter months (at 21.1°C), but took only three days in the current study performed during summer months (at 25.8°C). Likewise, *N. laxa* (La1-2) and *N. adansonii* (Ad4-11) took

an average of 12 and 15 days, respectively, to complete development in the winter study and only eight days and seven days in the summer study. Floral developmental rate decreased at all stages of development.

Stigma receptivity and pollen viability were first evaluated indirectly using manual pollination, recording fruit set. Because pollen from different individuals may germinate and grow at different rates on a particular stigma, stigma receptivity of each accession was tested using pollen from two different pollen donor accessions. Through pilot studies, pollen donors had been previously determined to be highly sexually compatible with the study accessions. Stigmas representing a full range of developmental ages (in days past *Even* stage of development) were pollinated using either intraspecific pollen (pollen from an accession of the same species) or interspecific pollen (pollen from an accession of a different species). For *N. adansonii*, only intraspecific pollinations were attempted because a highly compatible individual of a different species was not identified.

Fruiting success is reported in Tables 1.6 and 1.7. Table 1.6 reports fruiting success at each day of floral development. In all cases, three pollinations were attempted per day per species per cross type (intra- or interspecific). Table 1.7 reports the same data organized by developmental stage of each bud or flower at time of pollination. Flowers of *N. humifusa* were in *Open flower* stage on days zero and one, W*ilted* on days two and three, and *Senescent* on days four and five. Due to variation in stage durations within accessions of *N. laxa* and *N. adansonii*, multiple stages were observed on some days. For example, of the 12 flowers of *N. laxa* pollinated at *Open flower* stage, four were at the fifth day after *Even* stage, six at the sixth day, one at the seventh day, and one

at the eighth day. Similarly, of the three *N. adansonii* flowers pollinated on day seven, one was wilted, while two were senescent.

Pollen donor species had no effect on stigma receptivity in *N. humifusa* or in *N. laxa* in terms of day of pollination or in terms of developmental stage of the pollinated flower (Tables 1.6, 1.7). Stigmas of *N. humifusa* were receptive to pollination on zero and one day past *Even* stage of bud development, with 100% fruiting success both in intraspecific and interspecific pollinations. All receptive flowers of *N. humifusa* were in *Open flower* stage, while *Wilted* or *Senescent* flowers were not receptive. Stages prior to *Open flower* are not represented in this study because buds progressed from *Calyx* stage to *Open flower* in one day. Stigmas of *N. laxa* showed first signs of receptivity at one day past *Even* stage. Receptivity peaked on days four and five, and pollination was unsuccessful on day nine. In terms of developmental stage, *N. laxa* flowers showed 50% receptivity during *Closed corolla*, peaked at 100% during *Open flower*, and fell to 64% at *Wilted*. As expected, results show that receptivity is highest in both species during *Open flower*. *N. laxa* also exhibited high receptivity during later stages of *Closed corolla* and during *Partially open* stage. These stages were unavailable for analysis in *N. humifusa*. Finally, timing of receptivity in *N. adansonii* was analyzed using intraspecific pollen only. At *Closed corolla* stage, pollination success was limited to 33% fruiting success. Receptivity peaked on days four through six at corresponding developmental stages of *Partially open* and *Open flower* with 100% fruiting success. These results are similar to those seen with *N. laxa*.

Similar techniques were used to evaluate pollen viability in *N. humifusa* and *N. laxa* (Tables 1.8, 1.9). Differences were evident in the success of *N. humifusa* pollen in

fertilization of intraspecific stigmas (83% success) versus interspecific stigmas (56%). Pollination of intraspecific stigmas with pollen of *N. humifusa* (Hu9-4) showed 100% success on days zero through two and 67% success on days three through five. These results correspond to 100% success with pollen of open and wilted flowers and 33% success with that of senescent flowers. Successful pollination of interspecific stigmas of *N. humifusa* was limited to pollen from days zero through three with 100% success on days zero and one, 67% success on days two and three, and failure on days four and five. Successful interspecific pollinations correspond to 100% success with pollen from open flowers, 67% with wilted flowers, and 14% with senescent flowers. For *N. laxa*, pollination success was equal between intraspecific and interspecific pollinations. Peak pollination success was seen using pollen from open flowers with 85% fruit set. Success fell to 67% and 38% using pollen from wilted and senescent flowers, respectively. Fertilization potential of pollen from partially open flowers for these two species was not evaluated in this study because flowers progressed from *Closed corolla* to *Open flower* in a single day. Pollinating with pollen from partially open flowers would be preferable to using pollen from open flowers because pollen from partially open flowers would not likely have been contaminated by pollinator activity at this stage. Additional studies are needed with other species to evaluate pollen performance at the *Partially open* stage. Data shows that, as expected, pollen from open flowers provides maximum fertilization potential, but interestingly, pollen from wilted or senescent flowers can still be used successfully in some cases.

An attempt was made to evaluate stigma receptivity and pollen viability by chemical enzyme staining with 3-4.5 Dimethylthiazol-2-yl-2.5-diphenyl-tetrazolium

bromide (MTT). When in the presence of dehydrogenase enzymes, MTT solution changes from yellow to purple. Presence of these enzymes on stigmas suggests that stigmas are mature and will be receptive to pollination. Similarly, presence of dehydrogenase enzymes in pollen suggests that it is mature and viable (Rodriguez-Riano and Dafni, 2000). MTT has been successfully used to identify stigma receptivity and pollen viability in several genera such as *Oxalis*, *Iris*, and *Caesalpinia* (Luo et al., 2006, Sapir et al., 2005, Shi-Jin et al., 2004, Wang et al., 2005). If proven successful in the two studied *Nolana* species, this method could be used to verify timing of stigma receptivity and pollen viability in flowers of other *Nolana* species. This rapid technique would eliminate the requirement of a highly compatible mate when testing for stigma receptivity or pollen viability, and would significantly reduce the time involved in testing new species because the need to verify fruiting success would be eliminated. Unfortunately, the MTT testing technique proved to be an unreliable indicator of both receptivity and pollen viability. All tested stigmas of both species tested positive for the presence of dehydrogenase enzymes regardless of the age of the stigma (Figure 1.2). There were no clear differences in staining intensity or staining location in the analyzed stigmas. This is not consistent with our manual pollination data which shows a clear peak in receptivity in both *N. humifusa* and *N. laxa* during the *Open flower* stage of development. Likewise, no differences were apparent when staining pollen samples. Almost all pollen grains in all samples tested positive for the presence of dehydrogenase enzymes (Figure 1.2). MTT test for dehydrogenase as conducted is not an effective method for identifying stigmas and pollen of the appropriate developmental stage for use in hybridization in *Nolana*.

The three *Nolana* species studied showed similarities in timing of stigma receptivity when plants were manually pollinated and fruit set success was analyzed. As expected, visual floral stage was a better predictor of receptivity than chronological age after the *Even* stage of development. Despite clear differences in floral development schedules in these species, all exhibited a peak in stigma receptivity during the *Open flower* stage. Additionally, *N. laxa* and *N. adansonii* showed similar patterns of extended receptivity with moderate pollination success at *Closed corolla* and high success at *Partially open* stage. Similarities were also seen in timing of peak pollen viability. Pollen from open flowers was shown to be highly successful in pollinating both intraspecific and interspecific flowers. Based on these results, it is hypothesized that hybridizations in all *Nolana* species will be most successful when pollinations are performed during the *Open flower* stage of development using pollen from O*pen* flowers regardless of the duration of the species' complete floral development schedule. Additionally, extended receptivity at closed bud stages in *N. laxa* and *N. adansonii* suggest that pollination prior to anthesis may be an effective technique in *Nolana* breeding which would allow for controlled hybridization before there is a chance of contamination with foreign pollen.

Pollination success at a range of developmental stages

Levels of stigma receptivity, as measured by successful fruit set, were evaluated in flowers of each key visual stage of floral development in five *Nolana* species in August, 2005. *N. laxa* and *N. adansonii* were excluded from this study because detailed information about the timing of receptivity in these species was obtained in the previous receptivity study. *N. elegans* was omitted because of insufficient flowering.

Results are reported in Figure 1.3. As predicted, maximum fertilization success was seen in *Open flower* stage. Interestingly, fertilization success was achieved at high levels in most other stages as well. All five species exhibited a wide window of pollination success. *N. humifusa* and *N. plicata* showed high fruiting success at all floral stages. However, mericarps in two fruits resulting from pollinations of *N. plicata* at *Wilted* stage appeared to be non-viable as they were shriveled and tan colored rather than plump and black. *N. aticoana* and *N. ivaniana* showed high fruiting success in all stages except the earliest tested stage of *Calyx*. As in *N. plicata*, mericarps in one fruit resulting from pollination of *N. ivaniana* at the *Wilted* stage appeared to be nonviable. *N. rupicola* showed the narrowest window of pollination success with only *Closed corolla*, *Open*, and *Wilted* stages being highly successful in fruit formation.

Pollination at *Closed corolla* and *Partially open* resulted in nearly 100% fruit set in all species, and *Even* stage was nearly as successful. This finding shows that that pollination success can be achieved at sufficiently high levels for breeding at stages prior to *Open flower*. Buds at *Calyx* stage should be avoided because pollination success at this stage was variable between species, ranging from 100% in *N. humifusa* to 0% in *N. rupicola*. In practical terms, our findings have determined that for all species, buds at *Closed corolla* or *Partially open* stage can be hybridized successfully. Corollas and attached anthers may be pulled from the bud at *Closed corolla* or *Partially open* stage and pollination can be performed immediately. This technique allows for efficiency in performing hybridizations while avoiding risk of self-pollination or contamination by pollinators.

Construction of floral development keys

Data from the preceding studies were compiled to create floral development keys which can be used as quick visual reference to obtain species-specific information regarding durations of developmental stages and effective timing for manual pollinations (Figures 1.4 – 1.11). These keys are an original design developed to fit the needs of our *Nolana* research programs. Similar keys could be developed for other genera for use in other breeding and research programs. Each key has three components. Photographs of representative buds or flowers from each developmental stage are displayed with corresponding graphs of fertilization success at each stage. Above the photographs, are timelines illustrating durations of each developmental stage. Divisions in the timelines represent days of observation, with'0' representing the stage observed on the day prior to the start of data collection, and in all cases, corresponds with the *Calyx* stage of development. '1' represents the morphological stage observed on the first day of data collection. Data of pollination success was unavailable for inclusion in the *N. elegans* key, as was stage duration data for the *N. rupicola* key.

Development of these keys provides a valuable tool for use in both breeding and in studies of sexual compatibility. While results may be dependant on geographic location and weather conditions, keys provide a visual reference for researchers to quickly identify flowers of an appropriate stage for use in hybridizations to ensure maximum fertilization potential.

Conclusions

The floral development studies conducted with *Nolana* have successfully provided the necessary information for future breeding and hybridization studies in these

species with confidence that manual hybridizations are performed at the time of highest fertilization potential. The morphological stages of development corresponding with peak fertilization potential for individual flowers of each species have been determined. By visual inspection of the available pool of flowers at any time, those flowers appropriate for use in hybridizations may now be quickly identified.

The initial survey of developmental schedules in each species identified differences in the durations of developmental stages between and within species. Although the duration of morphological stages was different between species, their schedules were very similar with respect to correlation between morphological floral stage and pollination success. As expected, maximum fertilization success occurred in the *Open flower* stage in all studied species.

With the techniques used in this study, we were not able to determine precise timing of pollen dehiscence. Further studies are necessary to determine timing of this event. Our future studies of self-fertilization will identify whether species are self-compatible and whether the need exists for emasculation in controlled pollinations of *Nolana*.

We determined that high levels of pollination success can be achieved at developmental stages prior to anthesis. With these findings, hybridizations for breeding purposes can be performed with confidence that stigmas are pollinated at a receptive stage while employing techniques to reduce risk of contamination by pollinators.

Table 1.1 Species, UNH accession codes, collection information, and habit for *Nolana* plants used floral development studies.

species	UNH accession code[z]	Collection Location	Habit[y]
N. laxa	La1-2, La1-4, La1-5 La3-1, La3-2	11°58' S, 76°46' W, 670-700 masl, Peru, Lima, Los Condores Open pollinated progeny of accession L4 collected at 11°58' S, 76°46' W, 680 masl, Peru, Lima, Los Condores, 40 Km from coast	erect herbaceous perennial
N. humifusa	H28, Hu1-2 Hu9-4	12°11' S, 76°48' W, 170 masl, Peru, Lima, Lomas de Pachacamac Open pollinated progeny of accession H45 collected at 11°58' S, 76°46' W, 675 masl, Peru, Lima, Los Condores	herbaceous annual
N. plicata	P5, P7, P11	15°47' S, 74°21' W, 400 masl, Peru, Arequipa, Lomas de Atiquipa	herbaceous perennial
N. aticoana	A2, A3, A13	15°47' S, 74°21' W, 450-480 masl, Peru, Arequipa, Lomas de Atiquipa	herbaceous perennial
N. adansonii	Ad2-2, Ad2-3 Ad4-1, Ad4-11, Ad4-14	17°01' S, 72°02' W, 0-5 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo, few meters from sea shore	erect herbaceous perennial
N. ivaniana	Iv2-1, Iv2-3, Iv2-5	17°01' S, 72°02' W, 5-10 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo, 20-30 m from sea shore	erect herbaceous annual
N. elegans	Ele1, Ele2, Ele3, 051-3, 051-5	25°26' S, 70°26' W, 890 masl, Chile, Region II, Prov. Antofagasta, Cerro Perales, near Taltal	procumbent herbaceous annual
N. rupicola	Rup1, Rup2, Rup3	26°01' S, 70°36' W, 720-780 masl, Chile, Region III, Atacama, Prov. Chanaral, Parque Nacional Pan de Azucar. Las Lomitas	herbaceous perennial

[z]Herbarium vouchers housed at UNH Hodgdon Herbarium, Durham, NH and Field Museum of Natural History, Chicago, IL.
[y]Adapted from Tago-Nakazawa and Dillon, 1999.

Table 1.2 Average number of days (rounded to the nearest whole number) that buds or flowers of accessions of seven *Nolana* species remained in each of five designated stages of floral development. Values are based on observation of five buds per accession and three to five accessions per species. Differenct superscrips in the *Closed corolla* column indicate significant differences in duration of this stage between species at the 95% confidence level.

Species	*Even*	*Closed corolla*[z]	*Partially open*	*Open flower*	*Wilted*	Total duration
N. laxa	1	5^{d}	1	5	2	11
N. humifusa[z]	0	0^{b}	1	2	0	3
N. plicata[z]	0	1^{bc}	0	3	1	5
N. aticoana	0	0^{b}	1	3	1	4
N. adansoni[z]	1	9^{a}	2	2	1	14
N. ivaniana[z]	0	1^{bc}	0	3	0	5
N. elegans	1	2^{c}	1	3	2	7

[z]Statistically significant differences exist in the duration of one or more floral stages among accessions of *N. adansonii, N. humifusa, N. ivaniana,* and *N. plicata*. Details *N. adansonii, N. humifusa, N. ivaniana,* and *N. plicata* accessions are reported in Table 1.3.

Table 1.3 Average number of days (rounded to the nearest whole number) that buds or flowers of accessions of *N. adansonii, N. humifusa, N. ivaniana,* and *N. plicata* remained in each of five designated stages of floral development. Values are based on observation of five buds per accession. Differenct superscrips within columns indicate significant differences in duration of this stage within indicated species at the 95% confidence level.

	Accession	*Even*	*Closed corolla*	*Partially open*	*Open flower*	*Wilted*	Total duration
N. adansonii	Ad2-2	1	6^{a}	1^{a}	5^{a}	1	13
	Ad2-3	1	9^{b}	5^{b}	0^{b}	1	15
	Ad4-1	1	12^{c}	0^{c}	0^{b}	1	13
	Ad4-11	1	6^{a}	1^{a}	6^{c}	1	14
	Ad4-14	1	10^{b}	2^{a}	1^{b}	1	14
N. humifusa	H28	0	1	1	1^{a}	0	3
	Hu1-2	0	0	1	2^{b}	0	3
	Hu9-4	0	0	1	3^{c}	0	4
N. ivaniana	Iv2-1	0	1	0	1^{a}	0	2
	Iv2-3	1	1	0	1^{a}	0	3
	Iv2-5	0	1	0	6^{b}	0	7
N. plicata	P5	0	2	1	4^{a}	1	7
	P7	0	1	0	$3^{a,b}$	1	5
	P11	0	1	0	3^{b}	0	4

Table 1.4 Average closed corolla lengths (in millimeters ± standard deviation) beyond that of the calyx, in 15 to 25 buds in each of seven *Nolana* species upon observation at 24 h intervals in floral development tracking study. Observation day one represents the first day on which corolla length equaled or surpassed that of the calyx. *Open* represents the day on which corollas reached or surpassed the *Partially open* stage.

Species	Calyx length (mm)	Day of Observation												
		1	2	3	4	5	6	7	8	9	10	11	12	13
N. adansonii	5.7	0.0	1.2 ± 0.1	2.6 ± 0.6	4.5 ± 1.0	7.4 ± 1.7	11.1 ± 1.9	15.1 ± 3.1	17.9 ± 2.0	19.4 ± 1.2	19.9 ± 1.3	20.2 ± 1.7	20.5 ± 2.0	*open*
N. aticoana	11.7	*open*												
N. elegans	13.5	0.5 ± 0.1	5.7 ± 1.2	11.1 ± 2.0	*open*									
N. humifusa	7.8	*open*												
N. ivaniana	12.3	6.3 ± 3.8	*open*											
N. laxa	6.9	0.2 ± 0.4	2.8 ± 0.8	5.2 ± 2.5	8.7 ± 2.2	14.7 ± 3.3	20.6 ± 2.6	*open*						
N. plicata	13.2	8.5 ± 4.2	15.7 ± 3.2	*open*										

Table 1.5. Corolla colors and average corolla dimensions at *Open flower* stage of *Nolana* accessions used in floral development studies

		Corolla color[z]			Corolla size	
	Accession	Outer Corolla	Throat	Veins	Average Diameter[y] (cm ± st.dev.)	Average Depth[x] (cm ± st.dev.)
N. adansonii	Ad2-2	92A	94B	--------		
	Ad2-3	85C	85B	--------		
	Ad4-1	87D	--------	93C	2.0 ± 0.6	2.7 ± 0.3
	Ad4-11	92B	92A	--------		
	Ad4-14	87D	--------	93C		
N. aticoana	A2	94B	155C	89A		
	A3	91A	155C	89A	3.6 ± 0.4	2.2 ± 0.4
	A13	94B	155C	89A		
N. elegans	Ele1					
	Ele2	89B	155C	151A		
	Ele3	95B	155C	151A	4.5 ± 0.5	2.4 ± 0.5
	051-3	96B	155C	151A		
	051-5	95B	155C	151A		
N. humifusa	H28	92D	--------	88A		
	Hu1-2	92C	--------	88A	2.6 ± 0.6	1.3 ± 0.2
	Hu9-4	92B	--------	88A		
N. ivaniana	Iv2-1	91B	--------	--------		
	Iv2-3	91B	--------	--------	2.9 ± 0.5	1.9 ± 0.2
	Iv2-5	91B	--------	--------		
N. laxa	La1-2	88C	--------	88A		
	La1-4	88C	--------	88A		
	La1-5	87C	--------	87A	3.3 ± 0.4	2.7 ± 0.3
	La3-1	87D	--------	88A		
	La3-2	87C	--------	88A		
N. plicata	P5	94C	155C	89A		
	P7	94C	--------	89A	4.5 ± 0.5	2.9 ± 0.4
	P11	94C	155C	89B		
N. rupicola	Rup1	96A	155C	146D		
	Rup2	94A	155C	--------	5.4 ± 0.7	3.3 ± 0.3
	Rup3	96A	155C	146D		

[z]Corolla colors determined by R.H.S. Colour Charts.
[y]Calculated as the average diameter, at the widest point, of five fully expanded corollas per accession
[x]Calculated as the average depth. from the base of the receptical to the highest point of the corolla, of five fully expanded corollas per accession

Table 1.6. Stigma receptivity test: Success of pollination of *N. humifusa*, *N. laxa* and *N. adansonii* at a range of days past *Even* stage of bud development with pollen from flowers of a sexually compatible accession of the same species (intraspecific) or from flowers of a sexually compatible accession of a different species (interspecific). Values represent the number of successful pollinations out of three attempted pollinations per day, as measured by successful fruit set.

		Days past *Even* stage										
		0	1	2	3	4	5	6	7	8	9	total
N. humifusa	Intraspecific (Hu9-4 x Hu1-2)	3	3	0	0	0	0	--------	--------	--------	--------	6
	Interspecific (Hu9-4 x A2)	3	3	0	0	0	0	--------	--------	--------	--------	6
	Total	100%	100%	0	0	0	0	--------	--------	--------	--------	12
N. laxa	Intraspecific (La1-2 x La1-4)	0	1	2	2	3	3	2	1	1	0	15
	Interspecific (La1-2 x P5)	0	1	2	3	3	3	3	1	2	0	18
	Total	0	33%	67%	83%	100%	100%	83%	33%	50%	0	33
N. adansonii	Intraspecific (Ad4-11 x Ad4-14)	0	1	1	1	3	3	3	1	--------	--------	13
		0	33%	33%	33%	100%	100%	100%	33%	--------	--------	

Table 1.7. Success of pollination of *N. humifusa*, *N. laxa*, and *N. adansonii* stigmas over a range of floral development stages with pollen from flowers of a sexually compatible accession of the same species (intraspecific) or from flowers of a sexually compatible accession of a different species (interspecific). Values represent the number of successful pollinations versus the number of attempted pollinations per developmental stage, as measured by successful fruit set.

		Floral development stage							
		Even	*Closed corolla*	*Partially open*	*Open flower*	*Wilted*	*Senescent*	Fruits per pollination	Percent success
N. humifusa	Intraspecific	--------[z]	--------[z]	--------[z]	6/6	0/6	0/6	6/18	33%
	Interspecific	--------[z]	--------[z]	--------[z]	6/6	0/6	0/6	6/18	33%
	Total	--------[z]	--------[z]	--------[z]	100%	0	0	12/36	33%
N. laxa	Intraspecific	0/3	3/6	3/4	5/5	4/6	0/5	15/29[y]	52%
	Interspecific	0/3	3/6	4/4	7/7	3/5	1/5	18/30	60%
	Total	0	50%	88%	100%	64%	10%	33/59	56%
N. adansonii	Intraspecific	0/3	3/9	3/3	6/6	1/1	0/2	13/24	54%
		0	33%	100%	100%	100%	0		

[z]*Even*, *Closed Corolla*, and *Partially open* stages were not represented in buds at zero to five days past *Even* stage in *N. humifusa*.

[y]One flower senesced and fell prior to pollination.

Table 1.8. Pollen viability test: Success of pollination using pollen of *N. humifusa* and *N. laxa* from flowers at a range of days past *Even* stage of bud development to pollinate flowers of a sexually compatible accession of the same species (intraspecific) or flowers of a sexually compatible accession of a different species (interspecific). Values represent the number of successful pollinations as measured by fruit set out of three attempted pollinations per type of cross per day (with the exception of day nine in *N. laxa* in which only two flowers remained on the plant).

		Days past *Even* stage									
		0	1	2	3	4	5	6	7	8	Total fruits
N. humifusa	Intraspecific (Hu1-2 x Hu9-4)	3	3	3	2	2	2	--------	--------	--------	15
	Interspecific (A2 x Hu9-4)	3	3	2	2	0	0	--------	--------	--------	10
	Total	100%	100%	83%	67%	33%	33%	--------	--------	--------	25
N. laxa	Intraspecific (La1-4 x La1-2)	0	0	0	2	3	2	3	0	0/1	10
	Interspecific (P5 x La1-2)	0	0	1	3	3	1	1	1	0/1	10
	Total	0	0	17%	83%	100%	50%	67%	17%	0	20

Table 1.9. Pollination data from Table 1.6 arranged by floral stage of the pollen donor flowers at the time of pollination. Values represent the number of successful pollinations versus the number of attempted pollinations per developmental stage, as measured by successful fruit set.

		Floral development stage							
		Even	*Closed corolla*	*Partially open*	*Open flower*	*Wilted*	*Senescent*	Total	Percent success
N. humifusa	Intraspecific	--------[z]	--------	--------	6/6	3/3	6/9	15/18	83%
	Interspecific	--------	--------	--------	5/5	4/6	1/7	10/18	56%
	Total	--------	--------	--------	100%	78%	44%	25/36	69%
N. laxa	Intraspecific	0/3	0/6	--------[z]	5/6	1/2	4/8	10/25	40%
	Interspecific	0/3	1/6	--------	6/7	1/1	2/8	10/25	40%
	Total	0	8%	--------	85%	67%	38%	20/50	40%

[z] *Even*, *Closed corolla*, *Partially open* were not represented in buds at 0 to 5 d past *Even* in *N. humifusa*. *Partially open* was not represented in flowers of *N. laxa*.

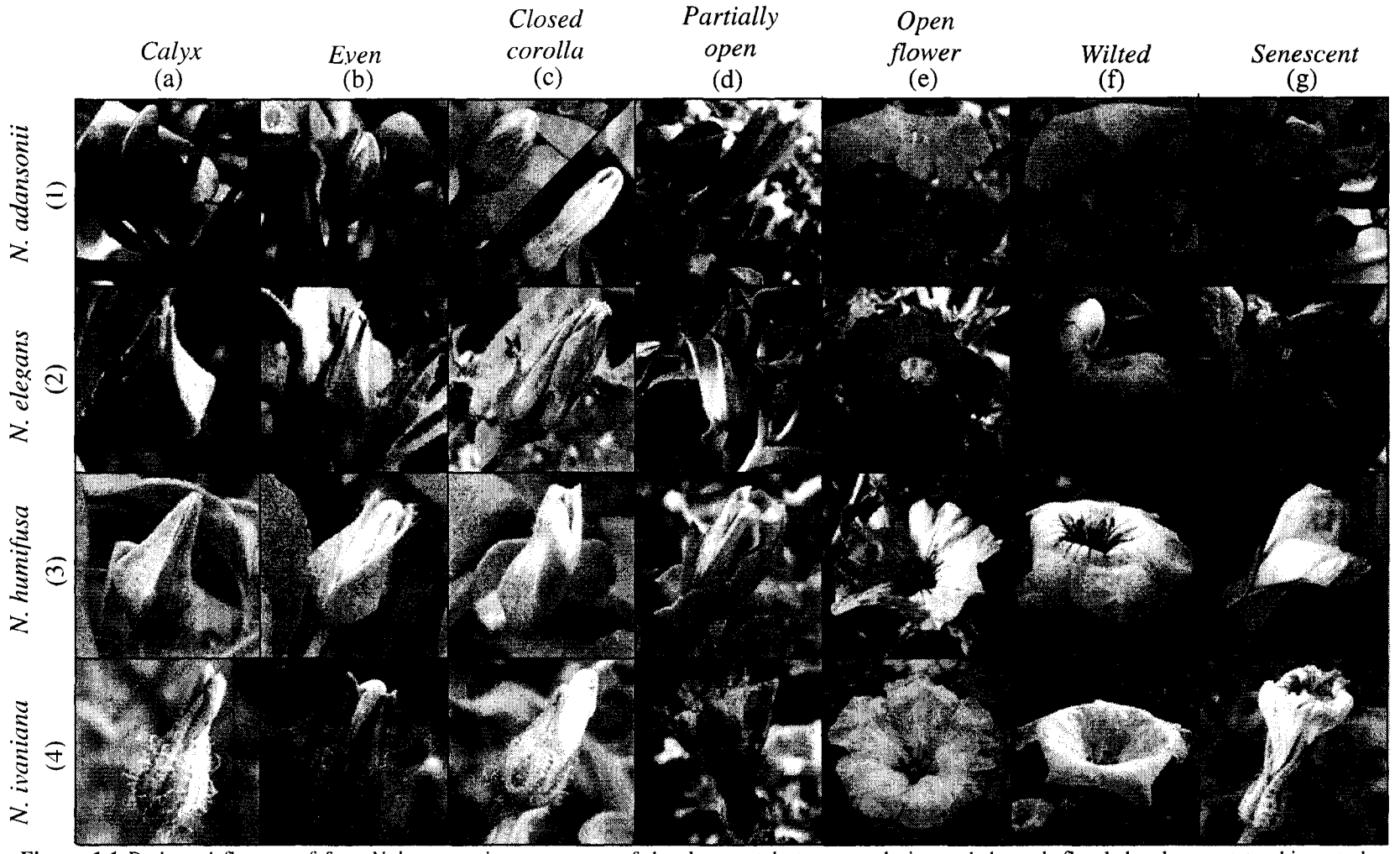


Figure 1.1 Buds and flowers of four *Nolana* species at a range of developmental stages as designated through floral development tracking study. Other species included in the study are not illustrated here, but exhibit bud morphologies similar to those of the species above. Buds of *N. adansonii* and *N. laxa* exhibit a prolonged *Closed corolla* stage; image 1-c illustrates buds representing early and late stages of *Closed corolla* in *N. adansonii*. Images are not to scale.

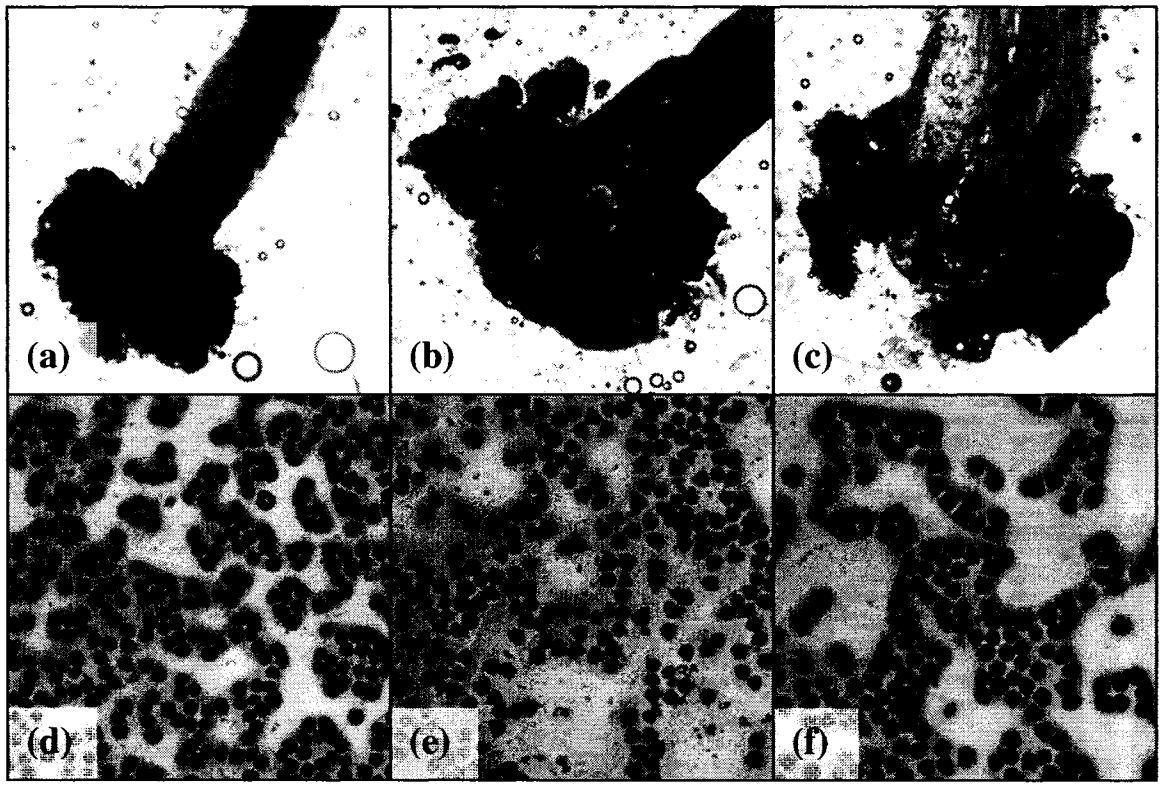


Figure 1.2 Stigmas and pollen of *N. laxa* excised and stained with MTT zero (a,d), four (b,e), and seven (c,f) days after *Even* stage of bud development. MTT was ineffective at differentiating stigma receptivity and pollen viability in this study. All samples reacted with similar color change regardless of age. Results were inconsistent with results of receptivity and viability tests using manual pollination.

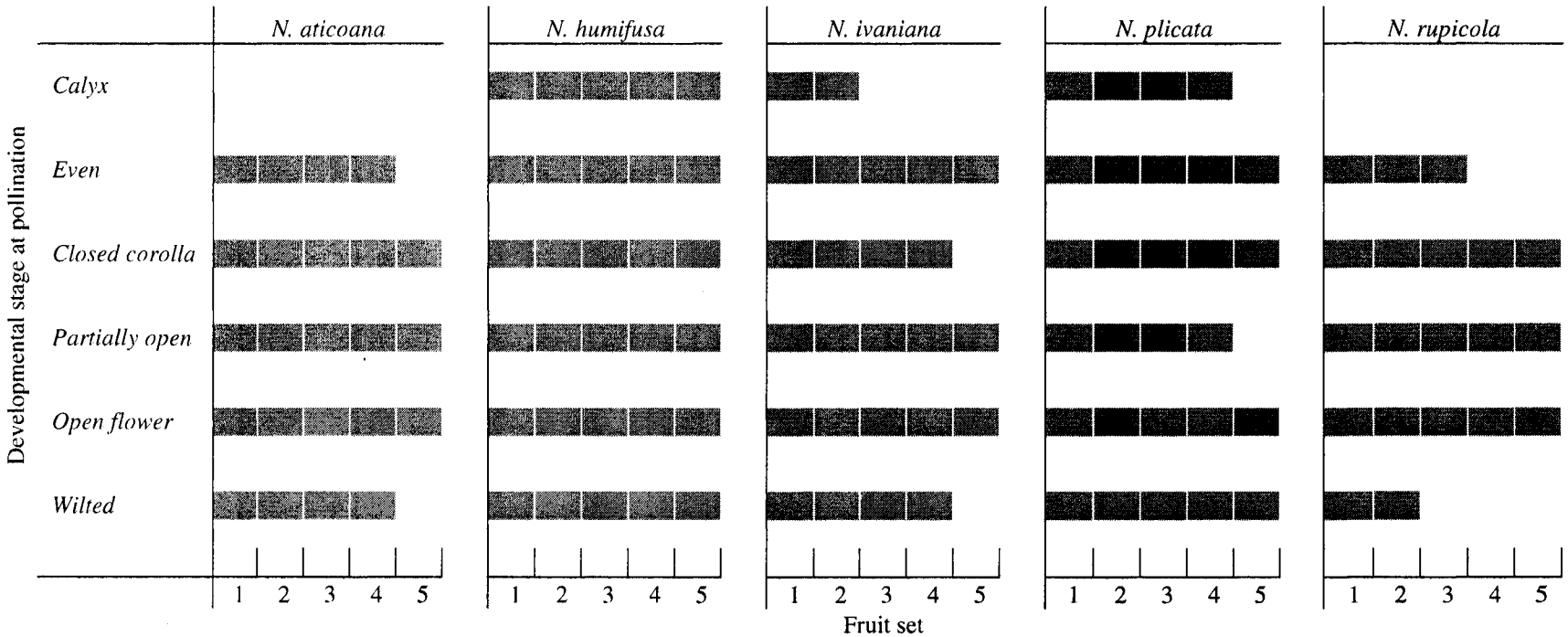


Figure 1.3 Success of pollinations of five *Nolana* species pollinated at each of six stages of floral development (*Calyx*, *Even*, *Closed corolla*, *Partially open*, *Open flower*, and *Wilted*). Bars represent the number of pollinations out of five attempted per developmental stage per species resulting in fruit set.

Nolana adansonii

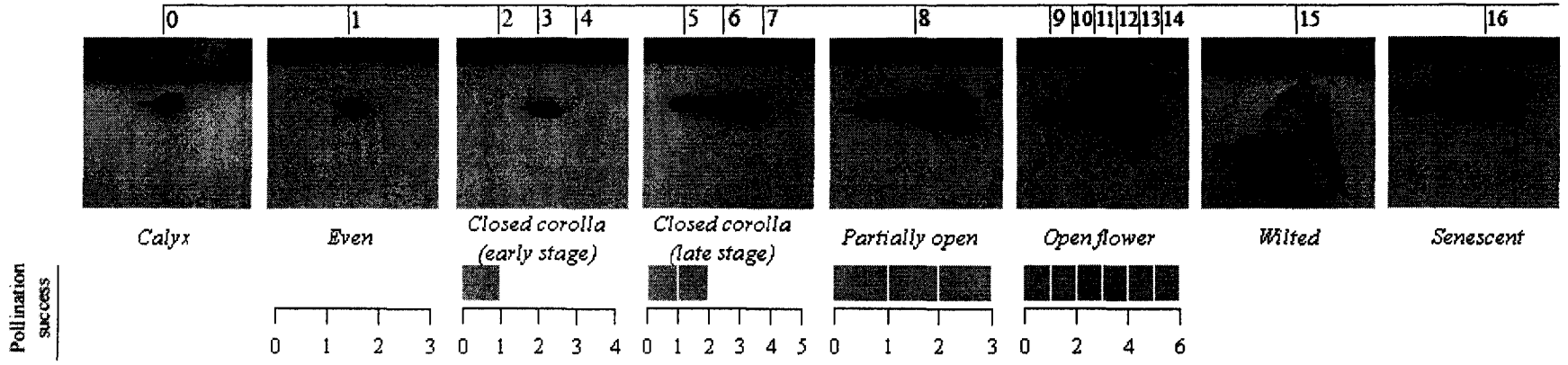


Figure 1.4. Floral development key for *N. adansonii* accession Ad4-11. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success of three to six buds per stage pollinated at *Even* through *Open flower* is reported below.

Nolana aticoana

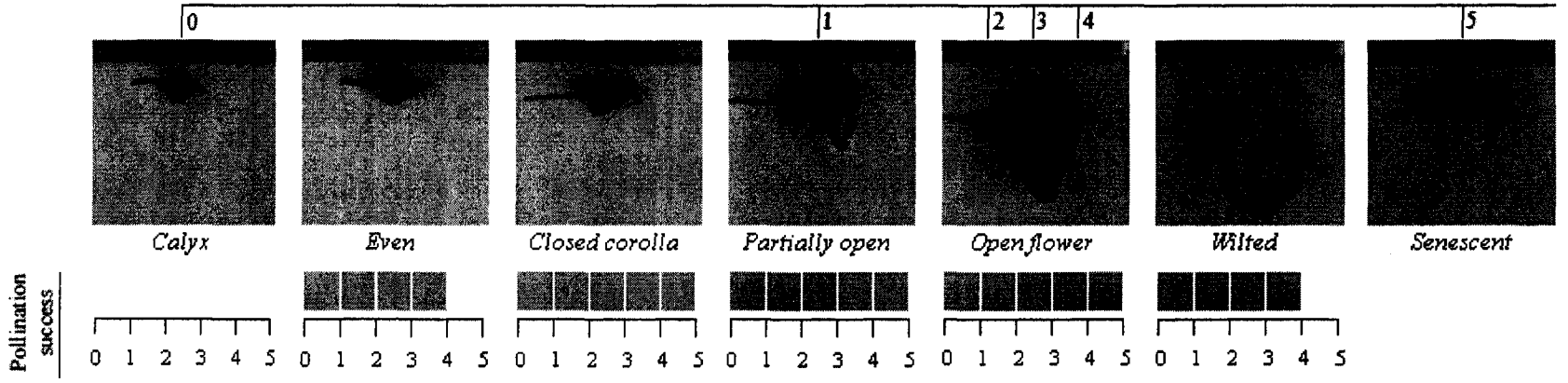


Figure 1.5. Floral development key for *N. aticoana*. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success of five buds per stage pollinated at *Calyx* through *Wilted* is reported below.

Nolana elegans

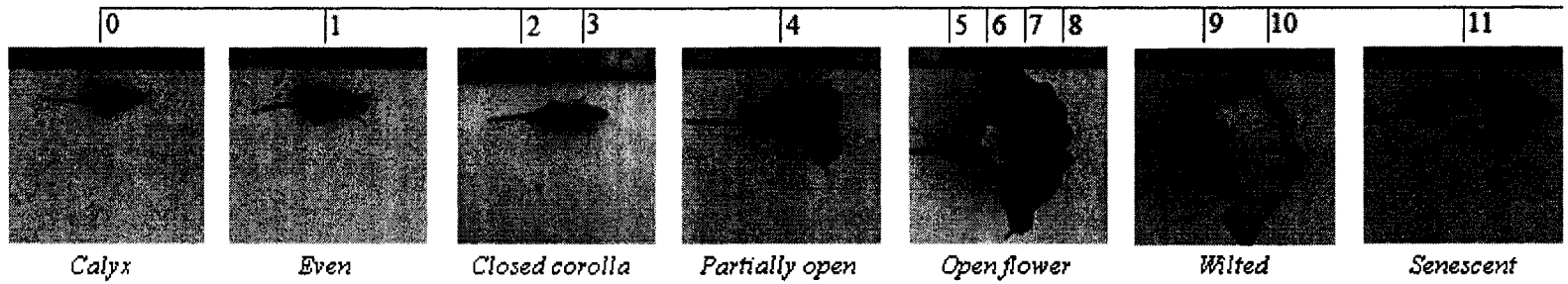


Figure 1.6. Floral development key for *N. elegans*. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success data is unavailable for this species.

Nolana humifusa

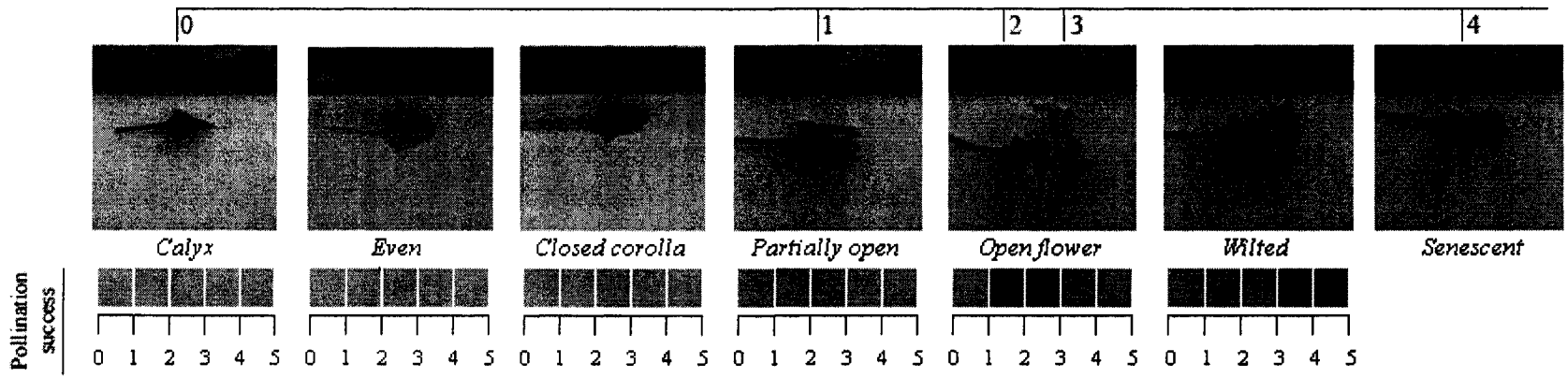


Figure 1.7. Floral development key for *N. humifusa*. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success of five buds per stage pollinated at *Calyx* through *Wilted* is reported below.

Nolana ivaniana

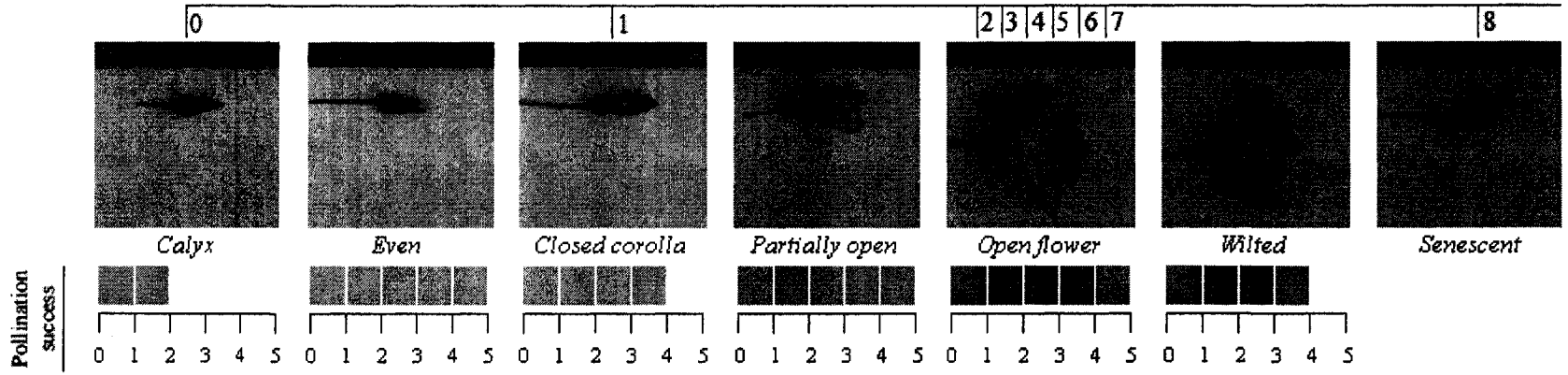


Figure 1.8. Floral development key for *N. ivaniana*. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success of five buds per stage pollinated at *Calyx* through *Wilted* is reported below.

Nolana laxa

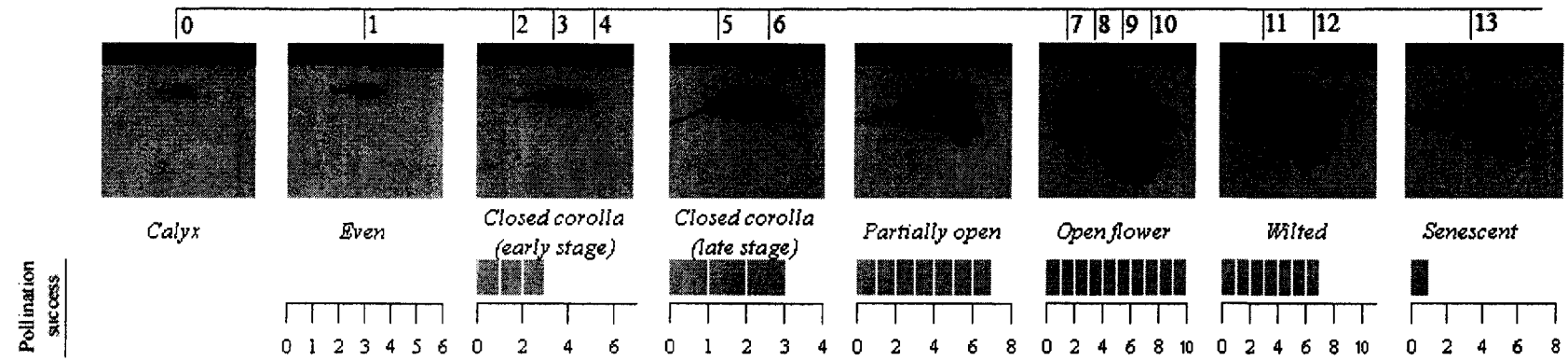


Figure 1.9. Floral development key for *N. laxa*. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success of four to eleven buds per stage pollinated at *Even* through *Senescent* is reported below.

Nolana plicata

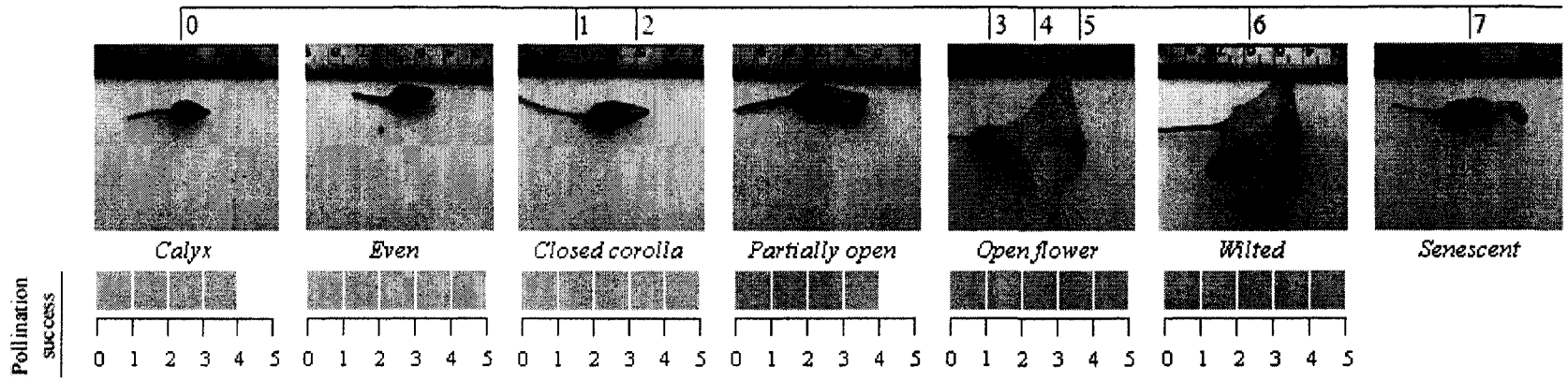


Figure 1.10. Floral development key for *N. plicata*. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success of five buds per stage pollinated at *Calyx* through *Wilted* is reported below.

Nolana rupicola

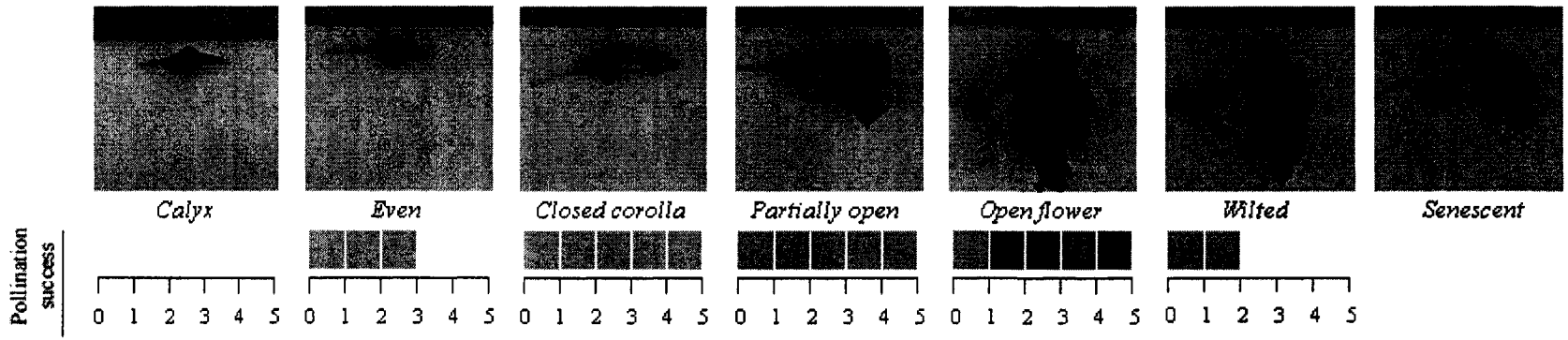


Figure 1.11. Floral development key for *N. rupicola*. Photos represent visual appearance of buds or flowers at each designated floral development stage. Timeline above photos indicates the average stage of buds/flowers observed on each day of the floral development study. '0' indicates stage at time of tagging, '1' indicates appearance one day later, increasing numbers represent appearance at 24 h intervals. Pollination success of five buds per stage pollinated at *Calyx* through *Wilted* is reported below.

CHAPTER 2

SEXUAL COMPATIBILITY BETWEEN EIGHT *NOLANA* SPECIES

Additional Index Words
interspecific, intraspecific, artificial hybridization, mericarp

Abstract

Artificial hybridizations (self- intra- and interspecific) were performed within and between eight species of *Nolana* L.f. (Solanaceae) (*N. adansonii*, *N. aticoana*, *N. elegans*, *N. humifusa*, *N. ivaniana*, *N. laxa*, *N. plicata* and *N. rupicola*). Plant material was grown from vegetative cuttings or seed collected at three *lomas* locations in the Peruvian desert in Peru and one *lomas* in the Atacama desert in Chile. Self-pollinations and all possible crosses, including reciprocals, were made between three to five accessions of each species. Crosses were made in replicates of five (or ten in self-pollinations), totaling 310 self-pollinations, 480 intraspecific pollinations, and 2,520 interspecific pollinations. Species were generally self-incompatible, with only one accession of *N. adansonii* and one accession of *N. ivaniana* exhibiting limited self-fertility as measured by fruit set. Intraspecific compatibility was high with most crosses resulting in greater than 80% fruit set. Success of interspecific hybridization was analyzed based on fruit set, mericarps per fruit, mericarp size, seed set, and seed germination. Differences in all these factors were found within seed parent species in response to hybridization with different pollen parent species. Reduction in interspecific fertility was generally seen as lower fruiting success and smaller mericarp size when compared to intraspecific hybridization, while the number of mericarps per fruit remained constant. A correlation was seen between

collection location and degree of presumed compatibility as measured by successful fruit set, with species collected in closer proximity to each other being more successful. Estimated seed counts were made by x-ray analysis of mericarps, revealing differences in seed counts between crosses. Hybrid seed was germinated producing hybrid seedlings from 22 unidirectional species pairs.

Introduction

Nolana L.f. is a large genus in the Solanaceae, endemic to the coastal Peruvian desert in Peru and Atacama Desert in northern-central Chile. Previously considered a unique family, Nolanaceae, due to its 5-carpelled gyonecium, the taxon was recently included as a genus within Solanaceae based on chloroplast DNA analysis (Olmstead and Palmer, 1992). The number of recognized species in the genus has ranged from 18 (Mesa, 1981; Hunziker, 2001) to 22 (D'Arcy, 1991), 63 (Johnston, 1936), 76 (Mesa, 1997), and the currently accepted 85 (Dillon et al., 2003). Of these, 37 species are endemic to Peru, 42 to Chile, four to both southern Peru and northern Chile, and one species is found only in the Galápagos Islands, Ecuador (Dillon, 2005).

The majority of species are found between 7°59' S and 33°21' S latitude, at 50-600 meters altitude, and within a few kilometers of the Pacific coast (Mesa, 1986; Dillon et al., 2003). Most species (70) are found in isolated patches of vegetation called *lomas* formations which are dependent on fog conditions during winter months (June - September). These areas flourish during El Niño years when the *lomas* experience unusually high rainfall and humidity (Dillon, 2005; Tago-Nakawaza and Dillon, 1999). *Nolana* are found in each of the 100+ *lomas* formations in Peru and Chile (Dillon, 2005). A *lomas* area may support a single *Nolana* species or up to ten species growing

sympatrically (Tago-Nakazawa and Dillon, 1999). Most species are narrow endemics, but few have wide distributions.

Nolana species range from herbaceous annuals to moderately woody perennial shrubs (Tago-Nakawaza and Dillon, 1999). Most species display showy flowers borne singly in leaf axils. Flowers are tubular-salverform to campanulate, infundibular, or rotate and range in size (1 cm diameter to 8 cm diameter) and in color (blues, purples, pinks, and whites) (Freyre et al., 2005).

The fruit of *Nolana*, the mericarp, is a unique derived character in the Solanaceae (Knapp, 2002). Fruit morphology varies by species with fruit reportedly consisting of two to 30 highly sclerified mericarps, mericarps being unilocellate or plurilocellate, and containing one to seven individual seeds each (Tago-Nakazawa and Dillon, 1999). Mericarp counts and seed counts are based on observations of field-collected open pollinated fruits. Each seed within a mericarp has an associated funicular plug which is displaced upon germination providing a canal through which the seedling exits the mericarp (Bondeson, 1986). Due to the plurilocellate characteristic of some mericarps, multiple seedlings may germinate from a single mericarp.

Cytological studies of *Nolana* are few, but suggest a chromosome count of x=12. 2n=2x=24 has been reported for *N. humifusa* (as *N. prostrata*) (Campin, 1925; Datta, 1933), *N. paradoxa* (as *N. atriplicifolia*) (Campin, 1925; Datta, 1933; DiFulvio, 1969), and *N. rostrata* (DiFulvio, 1984).

Reports of sexual compatibility within *Nolana* are very limited. Successful hybridization between *N. paradoxa* (as *N. atriplicifolia*) and *N. humifusa* (as *N. prostrata*), both artificial and spontaneous in cultivation, has been reported (Saunders,

1934). Cultivated *N. paradoxa* 'Bluebird' was successfully hybridized using wild *N. elegans*, *N. rupicola*, and *N. aplocaryoides* as male parents (Freyre et al., 2005). Self-compatibility in *N. paradoxa* and in *N. humifusa* has been reported (Saunders, 1934).

In the current study, sexual compatibility (including self-, intra-, and interspecific-compatibility) within *Nolana* is investigated. Included in the study are six Peruvian species collected from four *lomas* areas (*N. adansonii*, *N. aticoana*, *N. humifusa*, *N. ivaniana*, *N. laxa*, and *N. plicata*) and two Chilean species collected in a single area (*N. elegans* and *N. rupicola*). Self-pollinations, pollinations between accessions within each species, and pollinations between all possible combinations of species were performed by manual pollination. Sexual compatibility was evaluated based on fruiting success, mericarp and seed characteristics, and seed germination.

Materials and Methods

Plant material

Plant material included three to five accessions from each of eight *Nolana* species (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata*, and *N. rupicola*) vegetatively propagated and grown to maturity. Detailed collection information is reported in Table 2.1. Herbarium vouchers are housed at the Hodgdon Herbarium, University of New Hampshire, and at the Field Museum of Natural History, Chicago, IL. Photos of representative accessions of each species are displayed in Figure 2.1.

Species were selected to represent a range of geographic areas to which *Nolana* are endemic. Accessions were collected from three areas in Peru and one area in Chile,

with two species being collected from each area (Figure 2.2). *N. humifusa* and *N. laxa* are endemic to the northern ranges of *Nolana* habitat. *N. humifusa* was collected from coastal Lomas de Pachacamac, Lima, Peru and *N. laxa* from hillsides of Los Condores, Lima. The two locations are separated by approximately 50 Km. *N. humifusa* is an herbaceous annual with delicate trailing foliage and small (2-3 cm diameter) pale blue flowers marked with conspicuous dark purple veins. *N. laxa* is an erect herbaceous perennial with narrow slightly pubescent foliage and larger (3-4 cm diameter) deeply funnelform purple flowers. *N. plicata* and *N. aticoana* were collected approximately 500 km south of Lima at Lomas de Atiquipa, Ariquipa, Peru. The species were found growing separately with *N. aticoana* growing at a higher elevation (450-480 masl) than *N. plicata* (400 masl). Both species are herbaceous perennials. Flowers of *N. plicata* are 4-5 cm diameter and are a medium-pale blue in color. Foliage is trailing and pubescent. *N. aticoana* exhibits smaller (3-4 cm diameter) medium-blue flowers and glossy trailing foliage. *N. adansonii* and *N. ivaniana* were collected approximately 300 km south of Atiquipa, Peru on the seashore of Catarindo Beach, Arequipa, Peru. *N. adansonii* was found growing on the sand and hills in close proximity to the sea while *N. ivaniana* was found approximately 30 m inland. Both species are erect herbaceous perennials. *N. adansonii* is slightly woody with small cordate foliage. Flowers are small (2-3 cm diameter), purple, and deeply funnelform. *N. ivaniana* exhibits pale, narrow, pubescent foliage and small (2.5-3.5 cm diameter) pale blue flowers. *N. elegans* and *N. rupicola* are endemic to the southern range of *Nolana* habitat. *N. elegans* was collected at Cerro Perales, Chile, approximately 1600 km south of Arequipa, Peru. This species is an herbaceous annual with large (4-5 cm diameter) bright blue flowers and procumbent

pubescent foliage. *N. rupicola* was collected approximately 70 km south of *N. elegans* at Los Lomitas, Chile. *N. rupicola* is an herbaceous perennial with large (5-6 cm diameter) blue flowers. Foliage is procumbent and pubescent with a central rosette.

Plant material was maintained in an insect-exclusion double-poly hoop house located at the University of New Hampshire's Woodman Farm. Plants were grown in 25-cm diameter pots with Sunshine LA4 aggregate mix (SunGro Horticulture Inc., Bellevue, WA). Fertilization was constant with a 20N-4.3P-16.7K fertilizer at a maximum 150 $mg \cdot L^{-1}N$.

Terminology

For purpose of this study, the following definitions have been designated: *pollination* – pollination of a single flower; *accession-cross* or *cross* – cumulative pollinations between two unique accessions (unilateral; reciprocals excluded); *species-cross* – cumulative unidirectional crosses between two distinct species (unilateral; reciprocals excluded); *species pair* – cumulative bidirectional crosses between two unique species (bilateral; reciprocals included).

Self-pollinations

Ten flowers per accession were emasculated at a late bud stage prior to anthesis and pollen dehiscence. Dehiscent pollen from recently opened flowers on the same plant was manually applied by gently touching anthers to stigmas of emasculated buds. Self-pollinations were performed between December 2004 and February 2005.

Intraspecific hybridization

Manual pollination between three to five individuals within each of eight *Nolana* species was performed between January 2005 and March 2005. Five flowers were

pollinated for each possible cross within each species. Pollinations were performed on partially open or recently opened flowers by touching anthers of recently opened flowers of the pollen parent to stigmas of seed parent.

Interspecific hybridization

Three accessions from each of eight *Nolana* species were selected for use in the interspecific compatibility study (Table 2.1). Due to death of one individual of *N. adansonii* from diease before completion of the hybridization scheme, a fourth accession of this species was also included. Manual pollinations were performed between August 2005 and March 2006. Partially open or recently opened flowers of the seed parent were pollinated with pollen from recently opened flowers of the pollen parent. Five flowers were pollinated for each accession-cross. In total, 2,535 interspecific pollinations were performed representing nine accession-crosses in each of 56 species-crosses.

Harvest and storage

Fruits from successful hybridizations were left on the parent plant to mature. Fruits were harvested when mericarps became dark and, upon gentle squeezing of the fruit, were found to be loosened from the receptacle. Harvested mericarps were stored in paper coin envelopes in a desiccator at room temperature.

Data collection

Success of fruit set was recorded for each pollination. Counts were made of the number of mericarps per fruit from five fruits per intraspecific cross and from all successful interspecific crosses. Average mericarp mass of each cross was calculated from measurement of combined mass of up to 50 mericarps per intraspecific cross or all available mericarps per interspecific cross. Average mericarp diameter of each cross was

calculated from measurement of diameter of each mericarp at the widest area of the mericarp plug face on ten mericarps per cross. To obtain sufficient numbers of mericarps for size analyses, intraspecific crosses which had limited fruit set were repeated until at least five fruits were obtained.

Mericarps of each species and from each interspecific cross were x-rayed to estimate number of seeds per mericarp. Mericarps were x-rayed using the Faxitron MX20 Digital X-ray at Ohio State University's Ornamental Plant Germplasm Center. Random samples of twelve to 286 mericarps from each cross and species were x-rayed and digital images were recorded. Based on differences in contrast within the recorded images, counts were made of the number of presumed full seeds, and of cavities appearing empty or containing abnormally formed seed in each mericarp. Abnormal seeds were identified as those which did not appear to fully fill the cavity.

All mericarps from interspecific hybridizations were sown August 2006 in 128-plug trays with a 2:1 peat/perlite propagation medium at a distribution of one mericarp per cell. Mericarps were germinated under natural daylengths with an average temperature of 22.2°C and relative humidity of 73.4%. Number of seedlings germinating from each mericarp was recorded. Germinated seedlings were transplanted 15-cm pots and were grown in the greenhouse for future studies.

Data analysis

Hybridization data were analyzed in terms of fruiting success, mericarps per fruit, mericarp mass, and mericarp diameter by standardizing interspecific hybrid data to corresponding values obtained through intraspecific hybridizations. Crossability indices comparing the above variables were calculated for each species pair. 'Mericarps per fruit

indices' were calculated for each species-cross by first calculating a sub-index for each individual cross resulting from the average number of mericarps per fruit specific to the cross, divided by the average number of mericarps per fruit specific to that seed parent in intraspecific hybridizations. Sub-indices were adjusted to account for differences in number of fruits represented in individual crosses by multiplying the obtained value by the number of fruits represented in the cross. The sum of the adjusted sub-indices for the species combination was then divided by the total number of fruits represented in the species combination to obtain the two species' combination 'mericarps per fruit index.' Indices of mericarp mass, mericarp diameter, and seed set were developed using similar calculation.

A frequency distribution table of compatibility indices representing the combined effects of fruit set, mericarps per fruit, and seed set was produced using techniques adapted from McDade and Lundberg (1982). Crossability indices of zero indicate complete sexual incompatibility in interspecific hybridizations. Crossability indices of one indicate that values obtained through interspecific hybridizations are as high, or higher, than those of intraspecific hybridizations within the seed parent species. The frequency distribution table illustrates each species' hybridization success both as a seed parent and a pollen parent.

A numerical system was designed to represent distances between plant collection locations. Species pairs were assigned geographic separation values of one through five based on kilometers separating collection locations of parent material. Material was collected in four areas, with two species per area. Species within a collection area were assigned a separation value of one representing differing ecological niches within that

area or distances of up to 70 km between collection sites within an area. A value of two represents 280-500 km separation. A value of three represents 740-1010 km separation. A value of four represents 1200-1250 km separation. Most distantly separated species (1620-1690 km) were assigned a value of five (Figure 2.3).

Effects of pollen parent species on mericarp values were analyzed by ANOVA (Systat 10, SPSS Inc, 2000). Correlation between fruit set crossability indices and geographic separation values was calculated by Pearson's correlation (Systat 10, SPSS Inc, 2000).

Results and Discussion

Self-incompatibility

All species exhibited a high degree of self-incompatibility. Six species (*N. aticoana, N. elegans, N. humifusa, N. laxa, N. plicata,* and *N. rupicola*) failed to produce fruit upon self-pollination. One individual of *N. adansonii* (Ad2-3) and one individual of *N. ivaniana* (Iv2-2) exhibited limited self-compatibility, each with two out of ten pollinations resulting in fruit set. Fruits of Ad2-3 resulting from self-pollination contained fewer mericarps ($\bar{x}$ 7.3) than did those from intraspecific hybridizations involving the same seed parent ($\bar{x}$ 15.3). Number of mericarps per fruit was similar between fruits resulting from self- versus intraspecific pollination of Iv2-2, however, mericarps resulting from self-pollinations were smaller in mass and diameter (self: $\bar{x}$ mass: 3.3mg, $\bar{x}$ diameter: 1.4mm vs. intra: $\bar{x}$ mass: 8.3mg, $\bar{x}$ diameter: 2.4mm), suggesting reduced seed set.

Results show that while some self-compatibility does exist within *Nolana*, species are generally self-incompatible. Gametophytic self-incompatibility is a common trait within Solanaceae (de Nettancourt, 1997).

Based on these results, it is determined that emasculation prior to anther dehiscence is unnecessary in all but two of the studied accessions when used in our subsequent studies of intra- and interspecific sexual compatibility. This conclusion is supported by the facts that plants are housed in a pollinator-free greenhouse and that observed anther and stigma morphology does not facilitate unassisted self-pollination.

Intraspecific sexual compatibility

Using fruit set as indicator of sexual compatibility, species exhibited high rates of intraspecific sexual compatibility. Seven out of the eight studied species exhibited overall fruiting success upon intraspecific pollination at rates greater than 80% fruit set. *N. ivaniana* had unusually low fruiting success at 68%, possibly due to failing health of the plants at time of study.

Table 2.2 reports fruiting successes of the few intraspecific crosses that exhibited intraspecific incompatibility (failure of fruit set) or limited compatibility (low fruiting success). Of 96 attempted crosses, all but 23 crosses resulted in at least 80% fruit set. In most cases, incompatibility or low fruit set was unilateral, with reciprocals exhibiting high fruiting success. Of the 25 unique crosses performed within *N. laxa*, one cross (La3-1 x La1-5) failed to produce fruit. This incompatibility was unilateral with reciprocal cross (La1-5 x La3-1) exhibiting 100% fruit set. *N. elegans* exhibited one case of bilateral (Ele1 x 051-3; 051-3 x Ele1) and two cases of unilateral (Ele3 x Ele2 and Ele2 x 051-5) incompatibility. Reciprocals were compatible with 100% and 40% fruit set,

respectively. *N. ivaniana* exhibited one case of bilateral incompatibility (Iv2-1 x Iv2-3; Iv2-3 x Iv2-1) and one case of unilateral incompatibility (Iv2-1 x Iv2-5) with the reciprocal exhibiting 100% fruit set. All other crosses in all species were successful in producing at least some fruit, with degree of fruiting success ranging from 10% to 100% fruit set.

Germination of intraspecific hybrid seed was not evaluated in this study. Our previous studies have found *Nolana* seed to exhibit dormancy mechanisms which we have been unable to effectively overcome. Due to this dormancy, we expect germination rates to be low and not representative of true seed viability. Therefore, germination cannot currently be used as a measure of sexual compatibility in *Nolana*.

Species-specific fruit and mericarp characteristics

Table 2.3 summarizes intraspecific fruiting success, pollination success, mericarps per fruit, and mericarp sizes for each accession studied. Average values are also presented for each species.

Fruiting success and pollination success values represent compatible crosses only. These values illustrate intrinsic differences in levels of fertility between accessions of a single species. For example, *N. elegans* accession 051-5 exhibits only 60% fruiting success when crossed with compatible individuals of the same species, as compared to *N. elegans* accession Ele1 which exhibits 100% fruiting success. This information is critical to assessment of interspecific compatibility by comparison of fruiting success in interspecific hybridization to that in intraspecific hybridization. Based on this information, interspecific hybridizations using 051-5 as female parent may be deemed highly compatible if fruiting success near 60% is achieved. On the other hand, Ele1

would need to achieve near 100% fruiting success in order to be deemed highly compatible. Similarly, differences are evident in pollination success between accessions, suggesting that some accessions have an intrinsically weaker pollination potential.

As shown in Table 2.3, differences are evident both in number of mericarps per fruit and in mericarp size between seed parents, both within and between species. *N. rupicola* had the highest number of mericarps per fruit with an average of 18.3, and *N. laxa* had the fewest with an average of 3.8. Average mericarp mass ranged between species from 0.65 mg per mericarp in *N. adansonii* to 35.01 mg per mericarp in *N. plicata*. Average mericarp diameter ranged between species from 1.17 mm in *N. adansonii* to 4.62 mm in *N. plicata*. Mericarps of each species are displayed in Figure 2.4.

Interspecific sexual compatibility

Sexual compatibility exists between species if cross-pollination between them results in production of viable hybrid seed. Determination of viable seed production has been a challenge in our studies of *Nolana*. Although pollinations frequently result in successful fruit production, verification of existence of viable seed within those fruit has not been possible in many cases. Seed germination is commonly used as a simple measure of seed viability. In *Nolana*, however, germination has proven to be an unreliable measure due to our inability to effectively overcome seed dormancy. We have been unable to determine whether failure of hybrid seed germination is due to seed inviability or due to active seed dormancy. A second commonly used measure of seed viability is tetrazolium chemical enzyme staining. This method has also proven ineffective in *Nolana*. *Nolana* seeds are contained within highly sclerified mericarps.

We were unable to excise seed from the stony mericarps without inflicting injury to the seed which causes false positive results and nullification of enzyme staining. Since we could not directly document sexual compatibility by measure of viable hybrid seed production, we have measured levels of compatibility indirectly using values of fruit set, mericarps per fruit, mericarp sizes, and seeds per mericarp for each cross. By comparing these values as obtained through interspecific hybridization to values obtained through intraspecific hybridizations, degrees of compatibility between species can be inferred. In some cases, we have been able to verify existence of sexual compatibility between species by germination of hybrid seed. However, germination cannot be used as a measure of degree of compatibility, because germination rates are typically low and are unlikely representative of true rates of viable seed production.

Fruit set. Successful fruit set was used as an initial indicator of potential sexual compatibility between species. Results show that fruiting success in interspecific crosses is common in the *Nolana* species studied. Hybridization in 32 out of 56 species crosses resulted in at least one instance of successful fruit set. Bilateral compatibility was seen in ten species pairs, unilateral compatibility in 12, and bilateral incompatibility in six (Table 2.4).

Fruit set values for each species cross are reported in Table 2.4. Within each compatible species cross, successful fruit set occurred in a range of one to nine accession crosses out of a possible nine (Table 2.4, column *a*). Number of fruits developed in each successful cross ranged from one to five fruits out of a possible five, while total number of fruits developed per species cross ranged from three to 43 fruits out of a possible 45 (Table 2.4, column *b*).

Within bilaterally compatible crosses, differences are evident in levels of compatibility between reciprocals (Table 2.4). For example, *N. humifusa x N. aticoana* was highly successful with nine out of nine accession crosses and 43 out of 45 individual pollinations resulting in fruit set. Meanwhile, the reciprocal, *N. aticoana x N. humifusa* was only minimally successful with four out of nine accession crosses and five out of 45 pollinations resulting in fruit set. Other species pairs, such as *N. elegans* and *N. rupicola* with a total of 71 out of 90 successful pollinations, were highly successful in both directions of hybridization. In contrast, *N. elegans* and *N. ivaniana* with a total of seven out of 90 successful pollinations, were only minimally successful in either direction of hybridization. Unilaterally compatible crosses were generally only moderately successful with fewer compatible accessions and lower overall fruit set than was seen in many bilaterally compatible crosses. Few species exhibited bilateral incompatibility (Table 2.4). Interestingly, all six bilaterally incompatible species pairs included either *N. adansonii* or *N. rupicola* as a parent.

Differences in ploidy levels between species are being investigated as a possible cause incompatibility. Differences in ploidy levels have been shown to cause both bilateral and unilateral incompatibilities in the Solanaceae resulting from imbalances in ratio of endosperm contributions from male and female parents (Ehlenfeldt and Hanneman, 1988; Ortiz and Ehlenfeldt, 1992). This concept, termed Endosperm Balance Number, was introduced by Johnston et al. (1980) and is thought to be a major cause of incompatibility in plants. Additionally, the possibility of heteromorphic incompatibility is being investigated with studies of style length and pollen sizes.

Crossability indices based on fruit set were calculated for each species pair and are reported in Figure 2.5, column *a*. These indices relate fruit set of interspecific hybridizations to that of intraspecific hybridizations involving the same seed parent species. Indices are reported on a scale of zero to one, with an index of one indicating fruit set in interspecific hybridization was equal to or greater than that in intraspecific hybridization involving the seed parent species. With values obtained through intraspecific hybridization being designated as benchmarks to which hybrid values are compared, indices of one indicate no reduction in compatibility in hybrid crosses.

Mericarp characteristics. Fruit set may be used as an initial indicator of possible sexual compatibility between species however, it can be misleading in some cases. Successful fruit set does not necessarily indicate production of viable hybrid seed. Fruits may develop containing non-viable seed or may contain no seed at all. We considered several additional factors in our analysis of success of hybridizations in *Nolana*. Because we are unable to dissect the sclerified mericarps of the *Nolana* fruit to determine how many seeds are contained within, we have employed methods of indirectly evaluating mericarp contents. We have used counts of mericarps per fruit and measurements of mericarp mass and diameter to imply relative successes of interspecific hybridizations. By comparing these values between crosses and to values obtained through intraspecific hybridization, differences in hybridization success may be identified.

ANOVA was used to identify differences in values of mericarps per fruit, mericarp mass, and mericarp diameter within seed parents in response to pollination by different pollen parent species. Crosses involving *N. rupicola* as seed parent were

excluded from these analyses because this species showed compatibility with only one pollen parent species (*N. elegans*). Of the seven analyzed species, pollen parent species had a significant effect on mericarps per fruit in only one species, *N. elegans* ($P<0.05$) (Table 2.5). Five species showed some significant differences in mericarp mass in response to pollen parent species, while six showed significant differences in mericarp diameter (ANOVA $P<0.05$) (Table 2.5). Results suggest that seed parents tend to produce a consistent number of mericarps per fruit regardless of pollen parent species. However, differences in sizes of individual mericarps may be a reflection of differences in levels of sexual compatibility between species pairs. We hypothesize that mericarps with smaller mass and diameter contain fewer seeds than do larger mericarps produced by the same seed parent.

A more complete understanding of the magnitude and significance of observed differences in mericarp size values may be obtained by comparing values resulting from interspecific hybridizations to corresponding values representative of intraspecific hybridizations. Data obtained through study of intraspecific sexual compatibility were used as baseline data to which values obtained through study of interspecific sexual compatibility are compared. Decreases in mericarps per fruit, or mericarp size values of interspecific hybridizations compared to intraspecific hybridizations involving the same seed parent may be an indicator of lesser fertility of the interspecific cross regardless of successful fruit set. Existence of significant within-species differences necessitates the analysis of values obtained through intraspecific hybridization first on an accession-wise basis rather than a species-average basis, with species generalizations being made only after adjustment to account for within-species differences.

Crossability indices were calculated representing ratios of inter- to intraspecific values adjusted to account for intrinsic differences in fertility and mericarp values between accessions. Figure 2.5 reports crossability indices of fruit set, mericarps per fruit, mericarp mass, and mericarp diameter for all compatible species combinations (columns *a, b, c,* and *d*). Crossability indices range from zero, indicating complete sexual incompatibility in a species cross, to one indicating values obtained through interspecific hybridizations are as high, or higher, than those of intraspecific hybridizations within the seed parent species. Decreased fertility in interspecific hybridizations is apparent in some species crosses (such as *N. laxa* x *N. humifusa*) as a reduction in fruiting success (fruit index: 0.37) while number of mericarps per fruit and mericarp size remains comparable to intraspecific values (indices of 0.80-0.91). Alternatively, in other species crosses, such as *N. plicata* x *N. laxa*, apparent reduced fertility is displayed as a decrease in mericarp size with a mass index of 0.30 and a diameter index of 0.53, while number of fruits and mericarps per fruit remains unchanged (indices of 0.90 and 1.00 respectively). Indications of reduced fertility range between these two extremes in other species crosses.

Collective consideration of all factors provides a more thorough indication of reduced fertility than does any one factor alone. Six species crosses (*N. humifusa x N. laxa, N. laxa x N. plicata, N. laxa x N. ivaniana, N. plicata x N. rupicola, N. ivaniana x N. plicata,* and *N. elegans x N. rupicola*) performed nearly as well as intraspecific crosses in all measured factors with all four indices falling above 0.70. Based on our hypothesis that fruiting and mericarp characteristics are representative of seed set, it is predicted that seed set in these six interspecific crosses is nearly as high as that of intraspecific crosses

using the same seed parents. In contrast, two species crosses (*N. elegans x N. aticoana* and *N. elegans x N. ivaniana*) performed poorly in all measured factors with all four indices falling below 0.50. We hypothesize that these values represent a severe reduction in seed set as compared to intraspecific hybridizations.

Although values analyzed here provide evidence of reduced seed set, they do not confirm such a reduction. To confirm that these external measurements of fruiting success and mericarp characteristics can be used as a reliable measure of reduced fertility of a cross, we must confirm that mericarp size is correlated to the number of seeds contained within.

Seed counts by x-ray analysis. *Nolana* seeds are enclosed within a sclerified mericarp and are not easily removed. A mericarp may be crushed and broken apart to expose enclosed seeds, however this method is highly destructive resulting in nearly 100% seed death. In order to count seeds within intact mericarps, we used non-destructive x-ray analysis to view mericarp contents.

Number of individual mericarps analyzed per species or species cross ranged from 12 to 286. Based on differences in contrast within the x-ray images, estimates were made of the number of fully formed seeds and abnormal seeds or empty seed cavities within mericarps. The designation of *full seed* was made when image contrast within a mericarp cavity was bright, indicating the presence of dense tissue. The designation of *empty cavity* was made when contrast of a mericarp cavity was dark indicating vacant space, and that of *abnormal seed* was given when a mericarp cavity appeared to contain some dense tissue but it did not fill the cavity (Figure 2.6). Several mericarps were dissected to verify accuracy of designations based on contrast.

Table 2.6 summarizes results of x-ray analysis. Data are not available for mericarps with seed parent *N. laxa* because differences in contrast in mericarps could not be distinguished as described above. For other species, differences were evident in number of full seeds per mericarp between species crosses. The average number of full seeds per mericarp ranged from zero in *N. plicata* x *N. laxa* to 4.00 in *N. ivaniana* x *N. rupicola.* Overall, mericarps of interspecific crosses contained an average of 1.8 full seeds per mericarp. Most interspecific hybridizations resulted in mericarps containing a mix of full seeds and empty cavities or abnormal seeds. Only one cross (*N. ivaniana x N. aticoana*) resulted in mericarps with 100% cavities containing full seeds. The majority of mericarps were found to contain at least one full seed, although this was not the case with all crosses. 93% of mericarps from the cross *N. elegans x N. laxa* did not contain any full seeds. *N. elegans x N. ivaniana, N. adansonii x N. plicata*, and *N. adansonii x N. aticoana* also resulted in high percentages of mericarps without seeds (73%, 78%, and 86%, respectively).

Comparison of seed counts from interspecific hybridizations to those of intraspecific hybridization show that, on average, fewer full seeds were present in interspecific mericarps. Remarkable exceptions were seen in crosses involving *N. rupicola* and *N. aticoana* as seed parents. On average, interspecific crosses involving these seed parents resulted in higher seed set per mericarp than did intraspecific crosses. This discrepancy may be due to imbalances in representation of individual seed parent accessions in the analyzed intraspecific mericarps. Seed parent accessions may exhibit inherent differences in seed set potentials. However, the intraspecific mericarps used for

this analysis had been bulked without noting specific parentage, therefore these inherent differences cannot be identified and adjusted for.

Based on comparison of mericarp size indices and seed set ratios, we determined that mericarp size is not an effective indicator of seed set. Measurements of mericarp mass and diameter are not correlated to numbers of full seeds contained within mericarps or to total number of mericarp cavities.

Overall compatibility levels. Consideration of seed count data obtained through mericarp x-ray along with fruit set and mericarps per fruit data provides greater detail of potential levels of interspecific sexual compatibility than does either data set alone. Figure 2.5 illustrates seed count data alongside crossability indices. Column *e* reports seed set indices comparing percent mericarp cavities appearing to contain full seeds from interspecific crosses to that of intraspecific mericarps of the same seed parent species. Seed set indices provide detail of reduced fertility beyond that available from measurement of external fruiting and mericarp characteristics alone. *N. aticoana* x *N. humifusa* had a low fruiting index of 0.11 indicating limited compatibility, but x-ray analysis shows that mericarps contain numbers of full seeds comparable to those seen in intraspecific crosses, and the values of mericarps per fruit are comparable as well. In contrast, *N. plicata* x *N. laxa* had a high fruiting index of 0.90 and a high mericarps per fruit index of 1.00. However, no full seeds were seen within the mericarps despite the large size of the mericarps and the unusually high number of cavities observed within the mericarps (8.58 cavities per mericarp). It is clear that fertility levels cannot be estimated from any one factor alone, as reduced fertility is displayed as reduction in fruiting success, reduced seed set per mericarp, or as a combination of these factors. In many

cases, reduced fertility measured as low fruit set is compounded by low seed set per mericarp. In some cases, few mericarps are produced per fruit, further reducing our estimate of fertility.

A frequency distribution table (Table 2.7) illustrates compatibility between species calculated as the compounded effects of fruit set, mericarps per fruit, and seeds per mericarp. Crosses with higher compatibility are displayed toward the left side of the table nearing indices of 1.00. Compatibility decreases along the x-axis toward the right of the table, with incompatible crosses grouped at the far right with indices of zero. Each species' fitness as a seed parent and as a pollen parent is displayed. In this way, directional differences in compatibility levels of reciprocal crosses are evident, as are differences in species' compatibilities as a seed parent versus pollen parent. For example, *N. humifusa* showed compatibility with all seven other species when acting as seed parent, but was only successful in crosses with three species (*N. aticoana, N. laxa*, and *N. plicata*) when acting as pollen parent. *N. rupicola* showed compatibility as seed parent in crosses with only one species (*N. elegans*), but was successful as pollen parent in crosses with four species (*N. humifusa*, *N. laxa*, *N. ivaniana*, and *N. elegans*).

Table 2.8 illustrates distribution of crossability indices as grouped by geographic separation values. A geographic separation value of one indicates species were collected either at the same location or at locations separated by less than 70 km. Species within a single location may occupy differing ecological niches within that location. A separation value of two indicates collection at locations separated by 280-500 km, three indicates separation of 740-1,010 km, four indicates separation of 1200-1250 km, and five indicates collection locations are separated by distances of 1620-1690 km (Figure 2.3).

Results illustrate a correlation between geographic separation and compatibility. Species collected near to each other exhibit higher compatibility with an average crossability index of 0.44. As geographic separation increases, a higher percent of species pairs fail to produce hybrid fruit, and those that do tend to display much reduced fertility. Interestingly, not all species pairs fit this trend. *N. adansonii* and *N. ivaniana* were collected at a single location, just meters apart, but show no signs of inter-compatibility. In contrast, *N. humifusa x N. rupicola* were collected as the most distantly separated locations, but remain compatible with an index of 0.13.

Seed germination. Germination of hybrid seed is not a reliable measure of sexual compatibility in *Nolana* due to dormancy barriors. However, if germination occurs it verifies the existence of some level of sexual compatibility between species. We attempted to germinate all interspecific hybrid seed obtained through this study. Although germination rates were generally low, hybrid seedlings were obtained from 22 of the 32 species crosses for which fruit was produced (Table 2.9). In crosses with germination, rates ranged from 0.5% in *N. humifusa x N. laxa* to 62% in *N. elegans x N. aticoana* in terms of seedlings per mericarp. Overall, 13% germination was achieved. Germination rates do not necessarily reflect levels of compatibility. Germination rates were generally much lower than the predicted number of seeds within the sown mericarps as determined by x-ray analysis. These germination potentials are also reported in Table 2.9.

In Table 2.4, species pairs in bold print have been verified as sexually compatible by germination of hybrid seed. In most cases, compatibility in those species appearing to be highly compatible as implied by fruit set values was verified by seed germination. An

exception to this is the cross *N. rupicola x N. elegans*. Despite high fruiting success and seemingly high seed set, no seeds germinated from this cross. The reciprocal cross, however, was quite successful in germination with 106 seedlings obtained from 459 mericarps sown.

Interestingly, sexual compatibility was seen in species pairs representative of all levels of geographic separation. Natural hybridization has not been observed in the field in areas where species grow sympatrically (Tago-Nakazawa and Dillon, 1999). Therefore, successful artificial hybridization between species is a significant finding.

For the ten species pairs for which fruit set was successful but where germination failed, a definitive designation of sexual compatibility cannot be assigned. We cannot know whether failure of germination was caused by nonviable seed or seed dormancy. Germination of hybrid seed has provided verification of sexual compatibility between 18 species combinations previously not known to be compatible.

Conclusions

These studies of sexual compatibility within and between *Nolana* species have expanded the current knowledge regarding sexual compatibility within the genus. We found species to be generally self-incompatible, with limited self-compatibility seen in two species (*N. adansonii* and *N. ivaniana*). Intraspecific compatibility is strong within species with few exceptions. Interspecific compatibility is common. Fruit set was achieved in 32 out of 56 species crosses, and hybrid seedlings were obtained from 22 of these crosses.

Using fruit set as an initial indicator of possible compatibility between species, we have classified 24 species crosses as incompatible based on unsuccessful fruit production. Twelve of these species crosses were bilaterally incompatible and 12 were unilateral with reciprocals exhibiting various degrees of fruiting set success from 7% to 78%. In total, fruit set was successful in 32 out of 56 species crosses. Fruit set success ranged from 7% to 96%, with an average of 44% fruiting success out of all attempted pollinations.

X-ray analysis showed that mericarp size is not correlated to number of seeds contained within, although mericarps from interspecific hybridizations were generally found to be reduced in size compared with those from intraspecific hybridizations. We were surprised to find that even the largest mericarps often contained a small number of seeds or even no seeds at all. Measurement of mericarp mass and diameter cannot be used to estimate relative levels of seed set success. X-ray technology, however, has proven to be a valuable tool in analysis of sexual compatibility within *Nolana*. Overall 30% of the analyzed mericarps were found to contain no seeds. These findings illustrate the importance of seed set measurements in analysis of sexual compatibility. Consideration of fruit set alone may provide falsely high estimates of hybridization potentials.

We observed reduced compatibility in interspecific hybridizations as reduced fruiting success, reduced seed counts, and to a lesser degree, reduced number of mericarps per fruit as compared to values obtained through intraspecific hybridizations. Cumulative consideration of these factors provides us with estimations of compatibility potentials of species crosses. A range of compatibility levels was seen with the most successful crosses (*N. humifusa x N. plicata, N. humifusa x N. aticoana, N. humifusa x N.*

laxa, and *N. aticoana x N. plicata*) being approximately 80% as successful as intraspecific crosses involving the same seed parent.

Germination of hybrid seed has provided verification of sexual compatibility between *Nolana* species previously not known to be compatible. Previous to these studies, sexual compatibility was known to exist only between *N. paradoxa* and *N. humifusa, N. elegans, N. rupicola*, and *N. aplocaryoides*. Eighteen new species combinations have been verified compatible. Results of these studies are a significant contribution to the current knowledge of sexual compatibility within *Nolana*.

Table 2.1 Species, UNH accession codes, collection information, habit, and latitudinal range for *Nolana* plants used in sexual compatibility studies.

Species	UNH accession code[y]	Collection Location	Habit[x]	Latitudinal Range[x]
N. humifusa	H28, Hu1-2	12°11' S, 76°48' W, 170 masl, Peru, Lima, Lomas de Pachacamac	herbaceous annual	7°54'-13°05' S
	Hu9-4	progeny of accession H45 collected at 11°58' S, 76°46' W, 675 masl, Peru, Lima, Los Condores		
N. laxa	La1-2, La1-4, La1-5[z]	11°58' S, 76°46' W, 670-700 masl, Peru, Lima, Los Condores	erect herbaceous perennial	11°25'-11°42' S
	La3-1, La3-2[z]	progeny of accession L4 collected at 11°58' S, 76°46' W, 680 masl, Peru, Lima, Los Condores		
N. plicata	P5, P7, P11	15°47' S, 74°21' W, 400 masl, Peru, Arequipa, Lomas de Atiquipa	herbaceous perennial	15°22'-15°48' S
N. aticoana	A2, A3, A13	15°47' S, 74°21' W, 450-480 masl, Peru, Arequipa, Lomas de Atiquipa	herbaceous perennial	15°48' - 16°14' S
N. adansonii	Ad2-2. Ad2-3[z]	17°01' S, 72°02' W, 0-5 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo	erect herbaceous perennial	15°48' - 16°14' S (20°12' S)
	Ad4-1, Ad4-11, Ad4-14			
N. ivaniana	Iv2-1, Iv2-2[z], Iv2-3, Iv2-5	17°01' S, 72°02' W, 5-10 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo	erect herbaceous annual	15°55' S
N. elegans	Ele1[z], Ele2, Ele3[z], 051-3, 051-5	25°26' S, 70°26' W, 890 masl, Chile, Region II, Prov. Antofagasta, Cerro Perales, near Taltal	procumbent herbaceous annual	22°05' - 26°40' S
N. rupicola	Rup1, Rup2, Rup3	26°01' S, 70°36' W, 720-780 masl, Chile, Region III, Atacama, Prov. Chanaral, Parque Nacional Pan de Azucar. Las Lomitas	herbaceous perennial	23°30' - 26°32' S

[z]Individuals used in intraspecific hybridizations only. All others used in both intraspecific and interspecific hybridizations.

[y]Herbarium vouchers housed at UNH Hodgdon Herbarium, Durham, NH and at the Field Museum of Natural History, Chicago, IL.

[x]Adapted from Tago-Nakazawa and Dillon, 1999

Table 2.2 Percent fruit set for those intraspecific crosses resulting in less than 80% fruit set. Percent fruit set in the reciprocal crosses are also reported. Crosses not listed resulted in 80% to 100% fruit set.

Species	Cross	Percent fruit set of reported cross[z]	Percent fruit set of reciprocal cross[y]
N. adansonii	Ad2-2 x Ad4-14	20%	80%
	Ad2-3 x Ad4-14	40%	80%
	Ad2-2 x Ad4-1	40%	100%
	Ad2-3 x Ad4-11	40%	100%
	Ad4-1 x Ad4-11	60%	100%
N. elegans	Ele1 x 051-3	0%	0%
	Ele2 x 051-5	0%	40%
	Ele3 x Ele2	0%	100%
	051-3 x Ele1	0%	0%
	051-5 x Ele1	20%	100%
	051-3 x 051-5	40%	80%
	051-5 x Ele2	40%	0%
N. ivaniana	Iv2-1 x Iv2-3	0%	0%
	Iv2-3 x Iv2-1	0%	0%
	Iv2-1 x Iv2-5	0%	100%
	Iv2-2 x Iv2-5	20%	20%
	Iv2-5 x Iv2-2	20%	20%
	Iv2-2 x Iv2-3	20%	60%
	Iv2-3 x Iv2-2	60%	20%
	Iv2-3 x Iv2-5	60%	100%
N. laxa	La3-1 x La1-5	0%	100%
	La1-2 x La1-4	60%	100%
	La3-2 x La1-2	60%	100%

[z]Percent fruit set out of five pollinations for the listed cross.
[y]Percent fruit set out of five pollinations for the reciprocal of the listed cross.

Table 2.3 Fruiting data for artificial intraspecific hybridizations within eight *Nolana* species.

		Seed parent averages					Averages per species			
	Seed parent accession	Fruiting success as seed parent[z]	Fertilization success as pollen parent[y]	Average mericarps per fruit[x]	Average mericarp mass (mg)[w]	Average mericarp diameter (mm)[v]	Average fruiting success	Average mericarps per fruit	Average mericarp mass (mg)	Average mericarp diameter (mm)
N. humifusa	H28	100%	100%	4.9 ± 0.3	5.54	2.09 ± 0.17				
	Hu1-2	100%	100%	5.0 ± 0.0	9.35	2.78 ± 0.30	100%	4.9 ± 0.3	9.12	2.6 ± 0.43
	Hu9-4	100%	100%	4.8 ± 0.4	12.47	2.92 ± 0.21				
N. laxa	La1-2	90%	85%	4.7 ± 0.9	8.13	2.25 ± 0.39				
	La1-4	100%	80%	4.2 ± 0.9	7.76	2.15 ± 0.48				
	La1-5	90%	100%	3.1 ± 1.0	8.81	2.68 ± 0.59	91%	3.8 ± 1.0	8.02	2.39 ± 0.52
	La3-1	87%	100%	3.4 ± 0.9	4.70	2.2 ± 0.29				
	La3-2	90%	95%	3.5 ± 0.6	10.68	2.68 ± 0.49				
N. plicata	P5	100%	100%	2.9 ± 0.3	49.80	5.3 ± 1.19				
	P7	100%	100%	5.0 ± 0.0	28.88	4.8 ± 0.71	100%	4.1 ± 1.0	35.01	4.62 ± 1.05
	P11	100%	100%	4.5 ± 0.5	26.35	3.77 ± 0.50				
N. atticoana	A2	100%	90%	4.6 ± 0.5	28.41	3.4 ± 0.37				
	A3	90%	100%	4.9 ± 0.3	29.46	3.58 ± 0.39	97%	4.8 ± 0.4	25.77	3.47 ± 0.53
	A13	100%	100%	5.0 ± 0.0	19.45	3.42 ± 0.76				
N. adansonii	Ad2-2	65%	95%	15.1 ± 2.4	0.72	1.27 ± 0.24				
	Ad2-3	70%	95%	15.3 ± 0.9	0.59	1.07 ± 0.17				
	Ad4-1	90%	80%	14.4 ± 2.1	0.67	1.13 ± 0.21	82%	15.5 ± 2.0	0.65	1.17 ± 0.23
	Ad4-11	100%	75%	17.5 ± 1.6	0.62	1.21 ± 0.25				
	Ad4-14	85%	65%	15.4 ± 1.6	0.64	1.15 ± 0.24				
N. ivaniana	Iv2-1	100%	90%	6.6 ± 0.5	14.00	3.02 ± 0.53				
	Iv2-2	40%	60%	12.2 ± 1.8	8.32	2.39 ± 0.74	68%	8.4 ± 2.7	8.87	2.44 ± 0.64
	Iv2-3	60%	60%	6.3 ± 2.6	5.68	2.05 ± 0.35				
	Iv2-5	73%	40%	8.3 ± 2.0	7.49	2.29 ± 0.48				
N. elegans	Ele1	100%	60%	7.7 ± 5.4	6.06	2.04 ± 0.45				
	Ele2	93%	80%	10.6 ± 5.0	3.44	1.87 ± 0.26				
	Ele3	80%	100%	10.1 ± 5.2	7.65	2.00 ± 0.43	83%	10.7 ± 5.4	6.17	2.07 ± 0.40
	051-3	80%	87%	10.1 ± 2.8	9.07	2.33 ± 0.43				
	051-5	60%	73%	15.0 ± 5.4	4.64	2.11 ± 0.31				
N. rupicola	Rup1	100%	100%	18.7 ± 1.8	9.57	2.57 ± 0.66				
	Rup2	100%	100%	17.7 ± 2.3	8.29	2.26 ± 0.41	100%	18.3 ± 1.9	9.00	2.39 ± 0.59
	Rup3	100%	100%	18.5 ± 1.8	9.13	2.33 ± 0.65				

[z]Fruiting success as seed parent = percent fruit set resulting from five pollinations per cross between three to five individuals per species with the indicated individual acting as the seed parent. Self-pollinations and incompatible crosses are excluded.

[y]Pollination success as pollen parent = percent pollinations resulting in fruit set from five pollinations per cross between three to five individuals per species with the indicated individual acting as the pollen parent. Self-pollinations and incompatible crosses are excluded.

[x]Average mericarps per fruit calculated from counts of the number of mericarps comprising each of five fruits per cross except in the case of *N. ivaniana* where fewer than five fruits per cross were available ± standard deviation.

[w]Average mericarp mass calculated from the total mass of up to 50 mericarps per cross.

[v]Average mericarp diameter calculated from the diameters of up to ten mericarps per cross as measured at the widest point on the mericarp plug face ± standard deviation.

Table 2.4 Fruiting success in artificial interspecific hybridizations of *Nolana*. Hybridizations were performed between eight species, three accessions per species. Accessions were cross-pollinated in every possible combination including reciprocals. Five pollinations were made per cross. In total, nine crosses and 45 pollinations were made per species combination. Species combinations in bold were verified compatible by hybrid seed germination.

Bilaterally Compatible				Unilaterally Compatible				Bilaterally Incompatible			
	Crosses with fruit set (a)	Total fruits developed (b)	Geo. sep. value[z] (c)		Crosses with fruit set (a)	Total fruits developed (b)	Geo. sep. value (c)		Crosses with fruit set (a)	Total fruits developed (b)	Geo. sep. value (c)
N. elegans* x *N. rupicola	9	36	1	***N. humifusa* x *N. ivaniana***	8	35	3	*N. adansonii* x *N. ivaniana*	0		1
N. rupicola x *N. elegans*	9	35		*N. ivaniana* x *N. humifusa*	0			*N. ivaniana* x *N. adansonii*	0		
N. plicata* x *N. aticoana	9	39	1	*N. adansonii* x *N. plicata*	8	19	2	*N. adansonii* x *N. elegans*	0		3
N. aticoana* x *N. plicata	8	37		*N. plicata* x *N. adansonii*	0			*N. elegans* x *N. adansonii*	0		
N. ivaniana* x *N. laxa	9	34	3	***N. elegans* x *N. aticoana***	7	17	4	*N. adansonii* x *N. rupicola*	0		3
N. laxa* x *N. ivaniana	8	34		*N. aticoana* x *N. elegans*	0			*N. rupicola* x *N. adansonii*	0		
N. humifusa* x *N. laxa	9	38	1	***N. elegans* x *N. plicata***	7	13	4	*N. adansonii* x *N. laxa*	0		3
N. laxa* x *N. humifusa	5	15		*N. plicata* x *N. elegans*	0			*N. laxa* x *N. adansonii*	0		
N. humifusa* x *N. aticoana	9	43	2	***N. ivaniana* x *N. plicata***	5	19	2	*N. aticoana* x *N. rupicola*	0		4
N. aticoana* x *N. humifusa	4	5		*N. plicata* x *N. ivaniana*	0			*N. rupicola* x *N. aticoana*	0		
N. humifusa* x *N. plicata	9	39	2	***N. ivaniana* x *N. rupicola***	5	19	3	*N. plicata* x *N. rupicola*	0		4
N. plicata x *N. humifusa*	2	4		*N. rupicola* x *N. ivaniana*	0			*N. rupicola* x *N. plicata*	0		
N. laxa* x *N. plicata	8	32	2	***N. elegans* x *N. laxa***	5	18	5				
N. plicata x *N. laxa*	3	4		*N. laxa* x *N. elegans*	0						
N. aticoana x *N. laxa*	5	18	2	***N. humifusa* x *N. adansonii***	3	14	3				
N. laxa* x *N. aticoana	3	9		*N. adansonii* x *N. humifusa*	0						
N. adansonii x *N. aticoana*	4	10	2	*N. humifusa* x *N. rupicola*	3	14	5				
N. aticoana* x *N. adansonii	3	11		*N. rupicola* x *N. humifusa*	0						
N. elegans* x *N. ivaniana	1	3	3	***N. ivaniana* x *N. aticoana***	3	8	2				
N. ivaniana x *N. elegans*	1	4		*N. aticoana* x *N. ivaniana*	0						
				N. laxa x *N. rupicola*	3	6	5				
				N. rupicola x *N. laxa*	0						
				N. humifusa x *N. elegans*	2	3	5				
				N. elegans x *N. humifusa*	0						

[z]Geographic separation values assigned based on distances between collection locations of plant material used.

Table 2.5 ANOVA significance values of differences in mericarps per fruit, mericarp mass, and mericarp diameter in interspecific hybridizations in response to differences in pollen parent species. Values marked with asterisks indicate significant differences at 95% confidence intervals.

	Seed parent	Mericarps per fruit P	Mericarp mass P	Mericarp diameter P
N. humifusa	H28	0.129	0.333	0.000*
	HU1-2	0.171	0.039*	0.000*
	HU9-4	0.155	0.013*	0.000*
N. laxa	LA1-2	0.182	0.518	0.001*
	LA1-4	0.647	0.292	0.005*
	LA3-1	0.939	0.382	0.005*
N. plicata	P5	0.747	0.011*	0.000*
	P7	0.700	0.474	0.482
	P11	0.621	0.078	0.000*
N. aticoana	A2	0.834	0.205	0.000*
	A3	0.271	0.128	0.068
	A13	0.730	0.018*	0.000*
N. adansonii	AD2-2	-----	-----	-----
	AD4-1	0.779	-----	0.224
	AD4-14	0.057	0.292	0.613
N. ivaniana	IV2-1	0.062	0.024*	0.000*
	IV2-3	0.726	0.007*	0.000*
	IV2-5	0.121	0.387	0.008*
N. elegans	ELE2	0.008*	0.161	0.057
	051-3	0.000*	0.013*	0.001*
	051-5	0.000*	0.358	0.794
N. rupicola	RUP1	-----	-----	-----
	RUP2	-----	-----	-----
	RUP3	-----	-----	-----

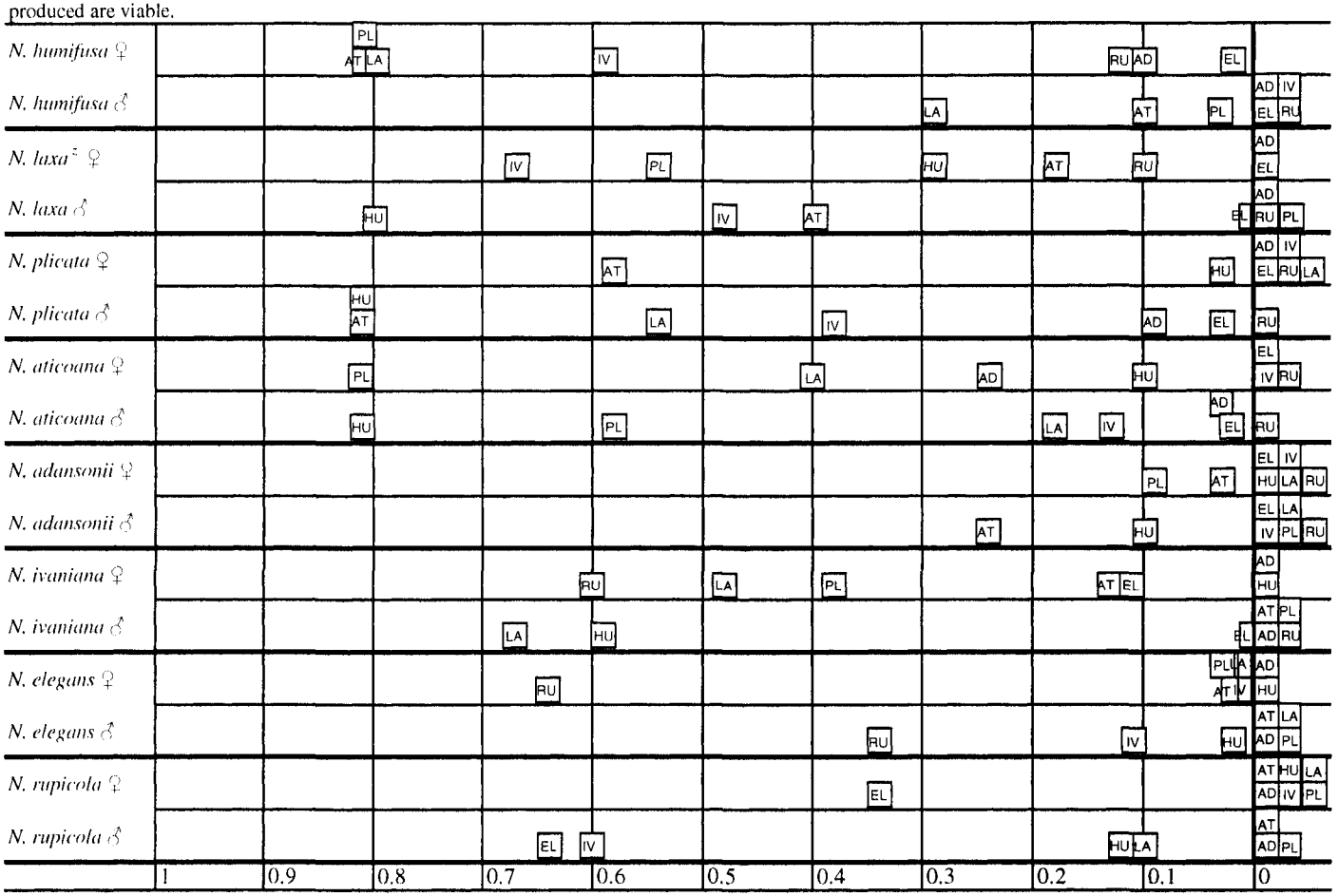
Table 2.6 Seed counts in mericarps developed through artificial interspecific hybridization or bulked intraspecific hybridizations as estimated from x-ray analysis.

	Number of mericarps analyzed	Average presumed full seeds per mericarp[z]	Average seed cavities per mericarp (full, empty, and abnormal)[y]	Percent cavities with presumed full seeds	Percent mericarps with one or more presumed full seeds
N. humifusa x *N. laxa*	180	2.58 ± 1.46	3.69 ± 0.95	70%	89%
N. humifusa x *N. plicata*	120	2.58 ± 1.73	3.98 ± 1.58	65%	90%
N. humifusa x *N. aticoana*	109	2.18 ± 1.55	3.69 ± 0.99	59%	83%
N. humifusa x *N. adansonii*	63	0.95 ± 0.94	2.86 ± 1.00	33%	67%
N. humifusa x *N. ivaniana*	182	2.01 ± 1.51	3.75 ± 1.15	54%	83%
N. humifusa x *N. elegans*	14	1.00 ± 1.78	3.07 ± 1.44	33%	71%
N. humifusa x *N. rupicola*	60	1.12 ± 1.44	3.25 ± 1.05	34%	60%
***N. humifusa* species seed**	**224**	**2.54 ± 1.40**	**4.27 ± 0.94**	**59%**	**93%**
N. plicata x *N. humifusa*	24	2.08 ± 1.41	4.50 ± 0.72	46%	92%
N. plicata x *N. laxa*	12	0	8.58 ± 3.23	0	0
N. plicata x *N. aticoana*	115	3.57 ± 2.15	4.97 ± 2.57	72%	97%
***N. plicata* species seed**	**89**	**5.03 ± 2.97**	**7.43 ± 3.03**	**68%**	**97%**
N. aticoana x *N. humifusa*	27	2.22 ± 1.45	3.78 ± 1.31	59%	89%
N. aticoana x *N. laxa*	100	2.48 ± 1.24	4.06 ± 1.56	61%	96%
N. aticoana x *N. plicata*	84	3.44 ± 1.80	4.31 ± 1.78	80%	96%
N. aticoana x *N. adansonii*	38	1.68 ± 1.04	4.08 ± 0.85	41%	87%
***N. aticoana* species seed**	**228**	**1.88 ± 1.81**	**3.94 ± 1.24**	**48%**	**64%**
N. adansonii x *N. plicata*	177	0.24 ± 0.46	1.07 ± 0.26	22%	22%
N. adansonii x *N. aticoana*	93	0.17 ± 0.46	1.05 ± 0.23	16%	14%
***N. adansonii* species seed**	**139**	**0.86 ± 0.39**	**1.06 ± 0.23**	**81%**	**84%**
N. ivaniana x *N. laxa*	214	3.05 ± 2.06	4.14 ± 2.04	74%	92%
N. ivaniana x *N. plicata*	88	2.77 ± 2.20	4.08 ± 1.67	68%	76%
N. ivaniana x *N. aticoana*	39	2.15 ± 1.55	2.15 ± 1.86	100%	87%
N. ivaniana x *N. elegans*	19	3.68 ± 1.77	4.74 ± 1.94	78%	95%
N. ivaniana x *N. rupicola*	78	4.00 ± 1.74	4.56 ± 1.70	88%	100%
***N. ivaniana* species seed**	**50**	**4.20 ± 1.80**	**5.16 ± 1.49**	**81%**	**94%**
N. elegans x *N. laxa*	27	0.07 ± 0.27	1.63 ± 0.74	5%	7%
N. elegans x *N. plicata*	35	0.63 ± 0.65	2.26 ± 1.27	28%	57%
N. elegans x *N. aticoana*	82	0.46 ± 0.61	2.09 ± 1.11	22%	40%
N. elegans x *N. ivaniana*	51	0.27 ± 0.45	1.49 ± 0.73	18%	27%
N. elegans x *N. rupicola*	286	1.82 ± 1.52	2.77 ± 1.97	66%	86%
***N. elegans* species seed**	**112**	**2.42 ± 1.80**	**3.97 ± 2.22**	**61%**	**94%**
N. rupicola x *N. elegans*	277	1.48 ± 1.08	1.94 ± 1.23	76%	85%
***N. rupicola* species seed**	**82**	**1.12 ± 1.00**	**1.62 ± 1.04**	**69%**	**77%**

[z]Designation of 'full seed' based on contrast in x-ray image. This value represents the number of cavities visible in the x-ray image appearing full. This may not be an accurate representation of viable seeds present. Value represents the average count of all analyzed mericarps +/- standard deviation. Value may represent multiple families within the indicated species pair.

[y]Seed cavities were identified in the x-ray images based on contrast. This count includes those cavities appearing to contain full seeds, those which appear empty, and those which appear to contain abnormal seeds. Value represents the average count of all analyzed mericarps +/- standard deviation. Value may represent multiple families within the indicated species pair.

Table 2.7 Frequency distribution table of crossability indices for artificial interspecific hybridizations between eight *Nolana* species.
Parent species are listed in the left column. Data is reported for each species performing as the seed parent (♀) or as the pollen parent (♂). Species with which the listed parent species were hybridized are represented by boxes containing the species abbreviation. The box's position on the index scale of one to zero on the x-axis indicates the species pair's degree of sexual compatibility based on fruit set, mericarps per fruit, and seed set. Compatibility decreases from left to right along the axis, with incompatible crosses being grouped at the right with indices of zero. Indices are calculated with the assumption that all seeds produced are viable.



AD=*N. adansonii*, AT=*N. aticoana*, EL=*N. elegans*, HU=*N. humifusa*, IV=*N. ivaniana*, LA=*N. laxa*, PL=*N. plicata*, RU=*N. rupicola*

[z]Indices of *N. laxa* are calculated from fruit set and mericarps per fruit indices only. Seed set indices are unavailable for *N. laxa*.

Table 2.8 Distribution of overall crossibility indices (CI) of interspecific hybridization of *Nolana* grouped by geographic separation value. Blocks represent individual species combinations (reciprocals represented separately). Higher crossability indices (approaching 1) indicate increased sexual compatibility as determined by fruit set, mericarps per fruit, and seed set. Here, a weak correlation is apparent between geographic separation and level of compatibility, with compatibility indices increasing as geographic separation decreases.

Geographic separation value[1]	avg. CI	
5	0.04	
4	0.01	
3	0.16	
2	0.24	
1	0.44	
Crossability index		1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0

[1]Geographic separation values are assigned to species pairs based on distance between collection locations. A value of 1 indicates species are separated by less than 68 km. Values increase as geographic separation increases, with the most distantly sepa

Table 2.9 Germination matrix of interspecific hybrid *Nolana* seed. Seed parent species are listed on the left, pollen parent species along the top. Cross values are reported where seed parent row and pollen parent column intersect. Reported values include number of mericarps sown, number of seedlings germinated, number of unique families represented in seedling populations, and estimated number of seeds in the mericarps sown as determined by x-ray analysis.

♀ \ ♂	*N. humifusa*	*N. laxa*	*N. plicata*	*N. aticoana*	*N. adansonii*	*N. ivaniana*	*N. elegans*	*N. rupicola*
N. humifusa	--------	221[z] 1[y] 1[x] (570)[w]	110 54 5 (284)	98 17 2 (214)	64 4 1 (61)	220 3 1 (442)	15 ng (15)	76 ng (85)
N. laxa	37 4 1 (nd)	--------	89 21 4 (nd)	29 3 2 (nd)	nf	105 4 2 (nd)	nf	21 ng (nd)
N. plicata	25 ng (52)	13 ng (0)	--------	179 8 5 (639)	nf	nf	nf	nf
N. aticoana	25 4 2 (56)	93 ng (231)	111 52 2 (382)	--------	60 19 3 (101)	nf	nf	nf
N. adansonii	nf	nf	176 ng (42)	106 ng (18)	--------	nf	nf	nf
N. ivaniana	nf	204 4 2 (622)	104 28 4 (288)	42 8 2 (90)	nf	--------	20 ng (74)	94 18 3 (376)
N. elegans	nf	32 1 1 (2)	40 15 3 (25)	109 34 6 (50)	nf	53 9 1 (14)	--------	466 101 7 (848)
N. rupicola	nf	nf	nf	nf	nf	nf	256 ng (379)	--------

[z]number of mericarps sown

[y]number of seedlings germinated, or ng=no germination, or nf=no fruits developed

[x]number of unique families represented by germinated seedlings

[w]estimated number of full seeds in sown mericarps as determined by x-ray analysis

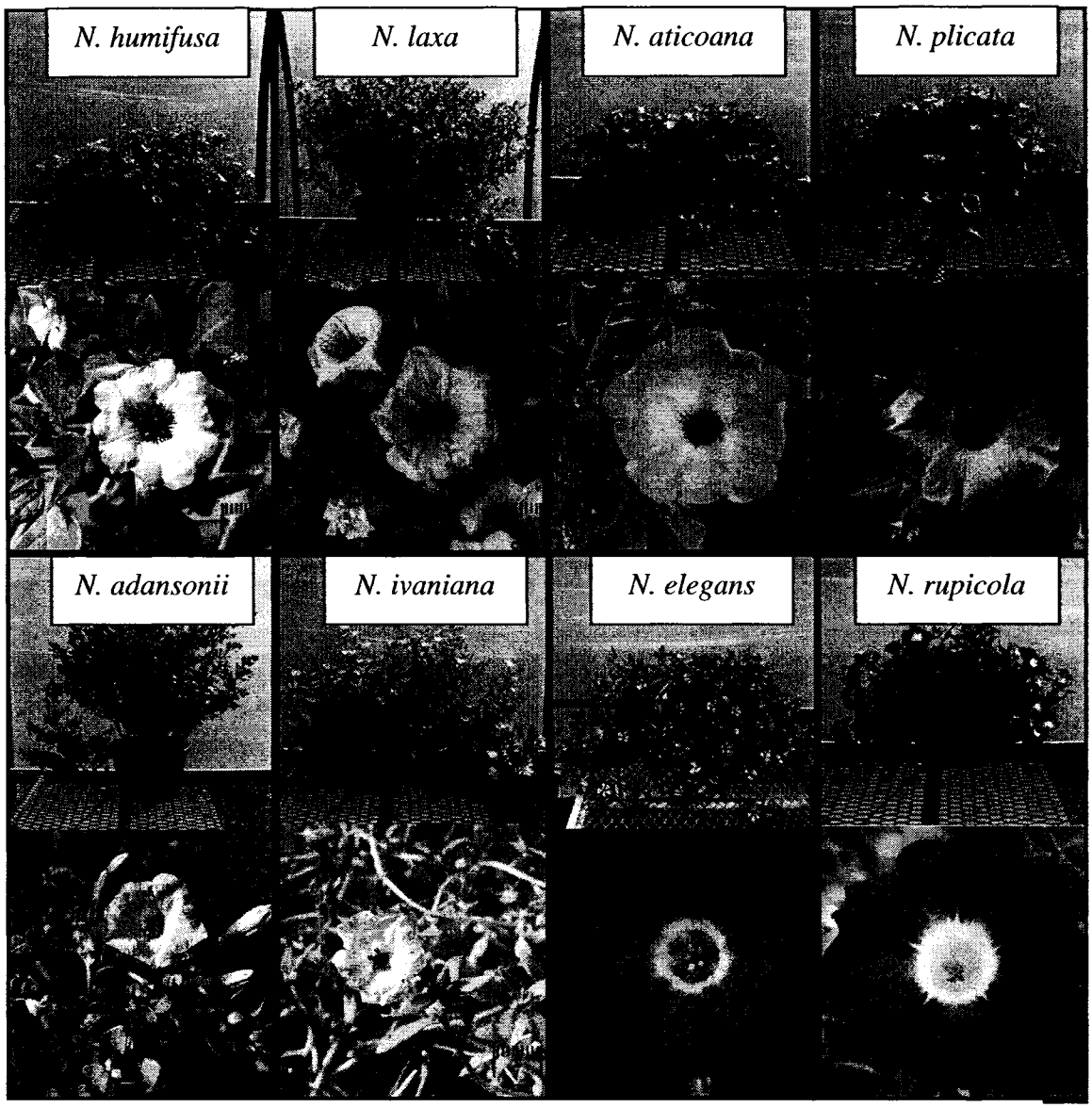
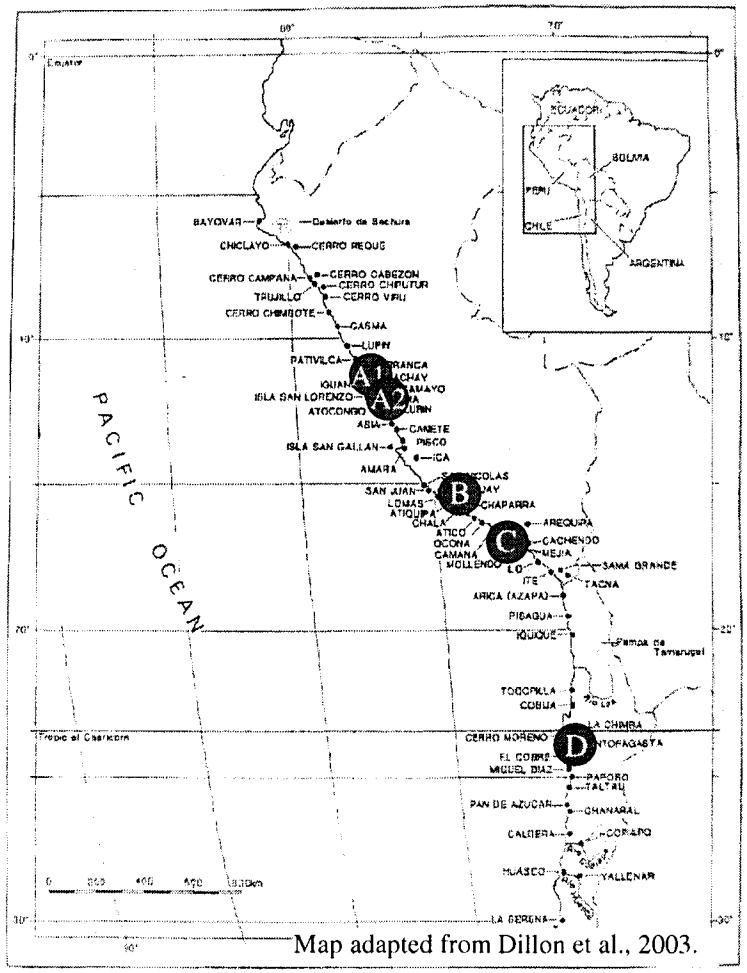


Figure 2.1 Plants and flowers of eight species of *Nolana* used in sexual compatibility studies. All plants are shown in 20-cm pots.



Map adapted from Dillon et al., 2003.

Figure 2.2 Collection locations of plant material included in sexual compatibility studies. Named locations indicate *Lomas* areas, where *Nolana* species are found seasonally when fog and rain are available. (A1, A2) collection sites of *N. humifusa* and *N. laxa*. (B) *N. plicata* and *N. aticoana*. (C) *N. adansonii* and *N. ivaniana*. (D) *N. elegans* and *N. rupicola*.

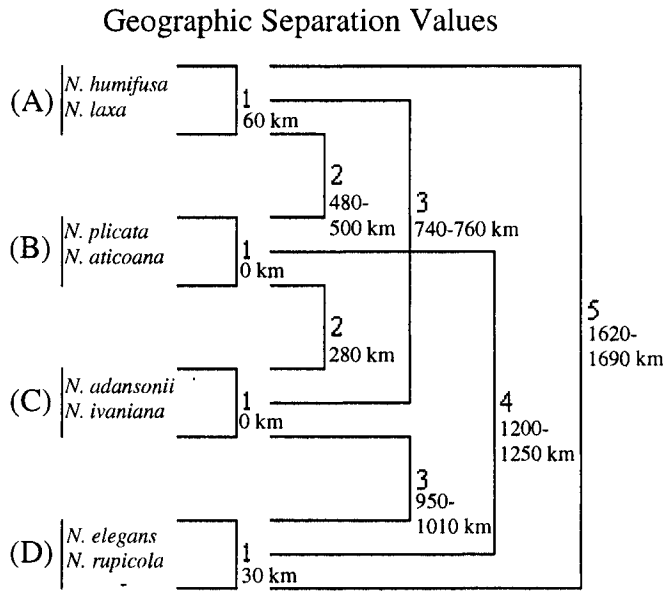


Figure 2.3 Geographic separation values of sites from which plant material used in sexual compatibility studies was collected. Actual distances between sites are also reported. .

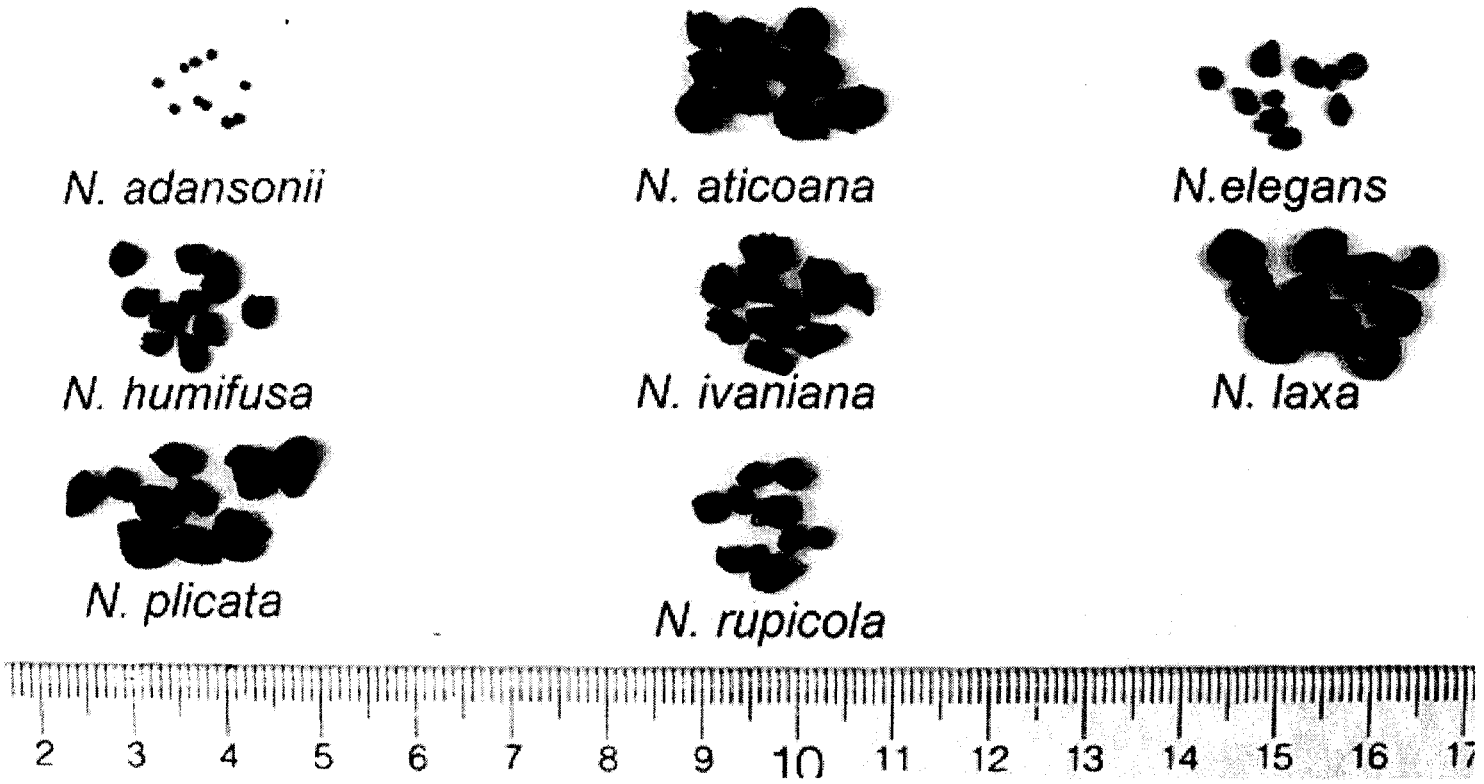


Figure 2.4 Mericarps of eight *Nolana* species.

Figure 2.5 Crossability indices for interspecific hybridizations between *Nolana* species. Indices represent performance of interspecific hybridizations in relation to intraspecific hybridizations in terms of (a) fruit set, (b) mericarps per fruit, (c) mericarp mass, (d) mericarp diameter, and (e) seed set. Indices range from zero to one, with an index of one indicating that the cross was comprable to intraspecific hybridization in terms of the given factor. Lower indices indicate inferior performace of interspecific hybridizations in relation to intraspecific hybridizations. Only crosses with successful fruit set are represented.

Cross	a	b	c	d	e
N. elegans x N. rupicola	1.00	0.86	0.73	0.77	0.75
N. rupicola x N. elegans	0.78	0.45	0.71	0.84	1.00
N. plicata x N. aticoana	0.87	0.96	0.88	0.76	0.71
N. aticoana x N. plicata	0.85	0.96	0.77	0.50	1.00
N. ivaniana x N. laxa	1.00	0.67	0.76	0.94	0.73
N. laxa x N. ivaniana	0.84	0.81	0.89	0.97	nd
N. humifusa x N. laxa	0.84	0.97	0.72	0.87	1.00
N. laxa x N. humifusa	0.37	0.82	0.80	0.91	nd′

Cross	a	b	c	d	e
N. humifusa x N. aticoana	0.96	0.99	0.65	0.55	0.86
N. aticoana x N. humifusa	0.11	1.00	0.64	0.83	1.00
N. humifusa x N. plicata	0.87	0.94	0.69	0.40	1.00
N. plicata x N. humifusa	0.09	1.00	0.41	0.69	0.41
N. laxa x N. plicata	0.79	0.70	0.77	0.92	nd
N. plicata x N. laxa	0.90	1.00	0.30	0.53	0.00
N. aticoana x N. laxa	0.41	1.00	0.88	0.91	1.00
N. laxa x N. aticoana	0.22	0.87	0.75	0.87	nd

Cross	a	b	c	d	e
N. adansonii x N. aticoana	0.24	0.81	nd	0.63	0.20
N. aticoana x N. adansonii	0.25	0.98	0.80	0.76	1.00
N. elegans x N. ivaniana	0.09	1.00	0.21	0.58	0.11
N. ivaniana x N. elegans	0.18	0.76	0.66	0.98	0.88
N. humifusa x N. ivaniana	0.78	0.98	0.57	0.84	0.79
N. adansonii x N. plicata	0.45	0.83	nd	0.57	0.28
N. elegans x N. aticoana	0.52	0.26	0.43	0.61	0.19
N. elegans x N. plicata	0.39	0.36	0.37	0.64	0.26

Cross	a	b	c	d	e
N. ivaniana x N. plicata	0.84	0.70	0.78	0.94	0.66
N. ivaniana x N. rupicola	0.84	0.76	0.66	0.91	0.95
N. elegans x N. laxa	0.55	0.74	0.49	0.67	0.03
N. humifusa x N. adansonii	0.31	0.98	0.57	0.83	0.37
N. humifusa x N. rupicola	0.31	0.97	0.51	0.80	0.44
N. ivaniana x N. aticoana	0.36	0.74	0.51	0.86	0.51
N. laxa x N. rupicola	0.15	0.76	0.32	0.82	nd
N. humifusa x N. elegans	0.07	1.00	0.49	0.51	0.39

(a) Fruit set index; (b) Mericarps per fruit index; (c) Mericarp mass index; (d) Mericarp diameter index; (e) Seed set index.
′ nd = data not available

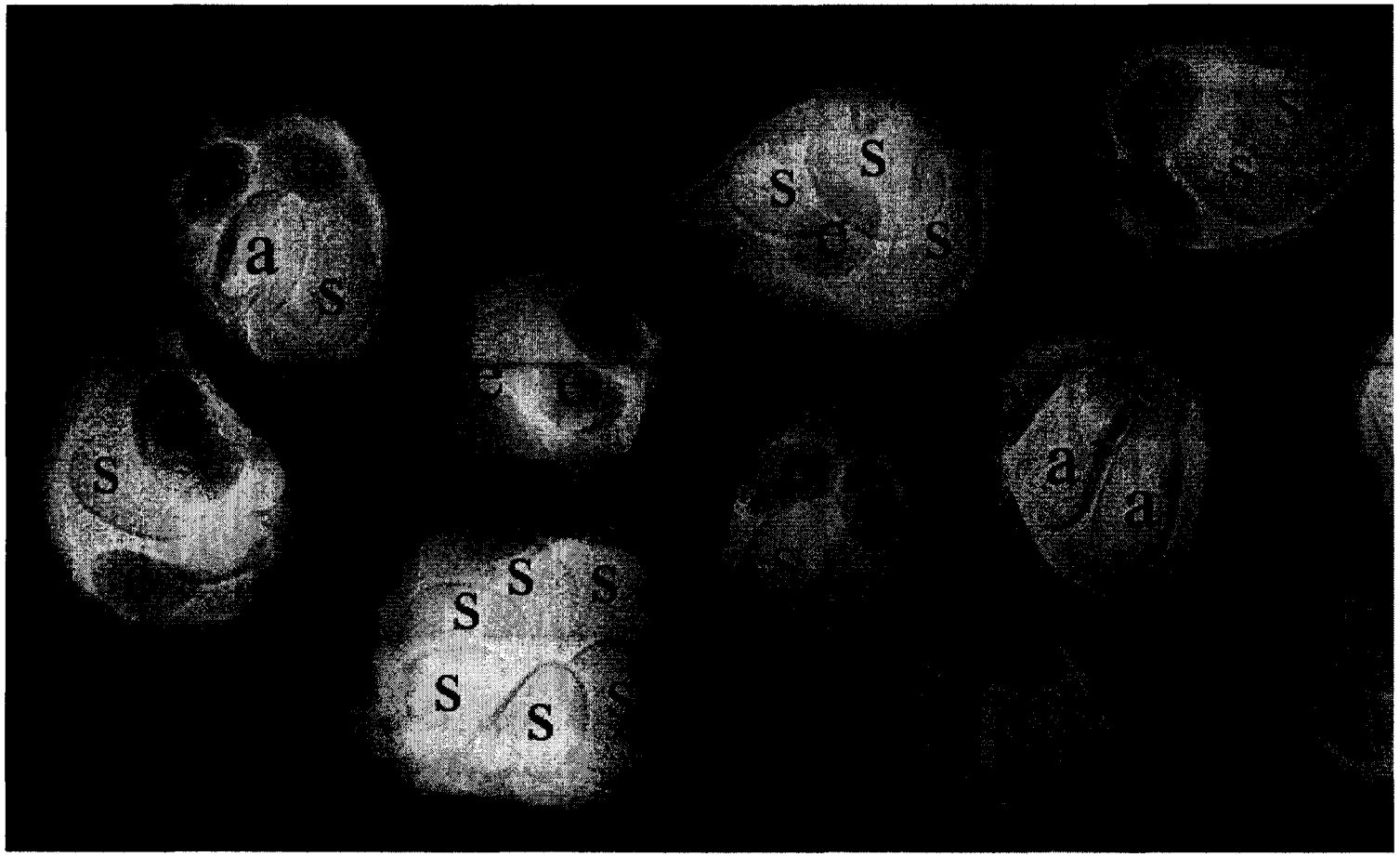


Figure 2.6 X-ray image of mericarps resulting from interspecific hybridization of *N. aticoana* x *N. adansonii*. Estimations of seed counts are made based on areas of differing contrast in the image. Cavities are labeled with 's' to designate a full seed, 'e' to designate an empty cavity, and 'a' to designate a seed which does not fill its cavity and therefore appears to be abnormally formed.

CHAPTER 3

SEED GERMINATION IN *NOLANA*

Additional Index Words
mericarp, funicular germination plug

Abstract

Nolana L.f. is a diverse genus of flowering plants native to coastal deserts of Peru and Chile. Low germination has been an obstacle to breeding and research efforts at the University of New Hampshire. *Nolana* fruits are comprised of unusual sclerified mericarps, each containing one or more embryos. Germination occurs with opening of funicular plugs on the mericarps, allowing radicle emergence. Under normal greenhouse conditions at UNH, germination success in eight *Nolana* species (*N. adansonii, N. aticoana, N. humifusa, N. laxa, N. ivaniana, N. plicata, N. elegans, N. rupicola*) frequently falls below 0.1 seeding per mericarp. We analyzed mericarp morphology, imbibition, and the effect of chemical and environmental germination treatments. Scanning electron microscopy revealed tracheid tubes in the funicular plugs. Mericarps were soaked in dye to track path and rate of imbibition, confirming that this occurs through the tracheid tubes, and that additional scarification is not required, although differences in rates exist between species. The following chemical treatments were unsuccessful in increasing germination: 0.1N HNO_3, 0.2% KNO_3, conc. H_2SO_4, 10 mM or 1*u*m ethephon. Gibberellic acid (1000 ppm) effectively increased germination in some species (up to 0.47 seedlings/mericarp). Older mericarps (stored dry for two years) had significantly higher germination than fresh mericarps (0.55 seedlings/mericarp).

Mericarps of *N. aticoana* stored for seven weeks at 35°C and 75% relative humidity showed significantly higher germination (0.36 seedlings/mericarp) than mericarps stored dry, or stored moist for one to six or eight to twelve weeks. Combinations of gibberellic acid and warm or high temperature treatment did not amplify positive results of individual treatments. Germination of seed cultured when immature did not surpass that of mature seed. X-ray analysis confimed existence of at least one seed within most mericarps. Up to 13 seeds were observed in single mericarps. Germination rates fall far below theoretical potentials based on x-ray seed counts. Our findings suggest failure of germination in *Nolana* is most often due to physiological dormancy rather than low seed set. Treatments with positive effects have been identified and will facilitate development of larger hybrid populations, thus increasing the efficiency of *Nolana* breeding and research programs.

Introduction

Nolana is the fifth largest genus in the Solanaceae, with 85 species currently described (Dillon et al., 2003). *Nolana* species range from herbaceous annuals to moderately woody perennial shrubs (Tago-Nakawaza and Dillon, 1999). Most species display showy flowers borne singly in leaf axils. Flowers are tubular-salverform to campanulate, infundibular, or rotate, and range in size (1 cm diameter to 8 cm diameter) and in color (blues, purples, pinks, and whites) (Freyre et al., 2005).

Nolana is endemic to the coastal Peruvian desert in Peru and Atacama Desert in northern-central Chile (Dillon, 2005). The majority of species are found between 7°59' and 33°21' S latitude, at 50-600 meters altitude, and within a few kilometers of the

Pacific coast (Mesa, 1986; Dillon et al., 2003). Most species (70) are found in isolated patches of vegetation called *lomas* formations which are dependent on fog conditions during winter months (June - September). These areas flourish during El Niño years when the *lomas* experience unusually high rainfall and humidity (Dillon, 2005; Tago-Nakawaza and Dillon, 1999).

Though yet unexploited, there is great potential to use *Nolana* in breeding programs to develop new ornamental cultivars. Desirable traits such as the large showy flowers, compact growth habit, and drought tolerance can be found throughout the genus. A *Nolana* breeding program was initiated at the University of New Hampshire (UNH) in 2001 with the goal of developing new *Nolana* cultivars for the ornamental plant industry. Since the program's inception, UNH has built a germplasm collection consisting of 22 *Nolana* species. Through controlled interspecific hybridizations, researchers aim to develop hybrid individuals possessing unique combinations of desirable morphological and physiological traits with increased ornamental value.

A significant obstacle to the progress of UNH's *Nolana* breeding program has been extremely low seed germination rates. The population size of the *Nolana* germplasm collection is limited due to low germination or complete failure of germination of wild seed. Consequently, genetic variation and desirable ornamental traits displayed by some species remain unavailable for integration into new cultivars. Low germination rates also have negative implications in later steps of the breeding process. Hybrid populations numbering in the hundreds are desirable to increase likelihood of obtaining an individual with a desirable combination of inherited traits. Because germination rates in *Nolana* are low, unusually high numbers of hybridizations must be

performed to generate enough seed to produce hybrid populations of acceptable size. The tasks of performing manual hybridizations, harvesting fruits upon maturity, and preparing hybrid seed for sowing require considerable time and effort. Efficiency of the breeding program is therefore hampered by low seed germination rates.

Storage of seed is often relied upon as a convenient and space-efficient method of preserving plant germplasm for future use. Low germination rates of *Nolana* seed prevent utilization of this resource-saving technique. In order to preserve genetic material of *Nolana* for future use, plant material must be maintained alive and propagated vegetatively. This technique requires large amounts of greenhouse space and effort, and results in a constant threat of loss of material due to disease infection and death.

Studies of sexual compatibility also rely on seed germination. By definition, two plant species are sexually compatible only if fusion of the species' gametes results in the development of viable seed. Successful germination of hybrid seed is therefore a definitive indicator of sexual compatibility. Without a complete understanding of the seeds' germination requirements, this test of sexual compatibility cannot be effectively utilized. Currently, only two reports relating to sexual compatibility in *Nolana* have been published (Saunders, 1934; Freyre, et al., 2005). At UNH we are conducting controlled hybridization studies to further document sexual compatibility between *Nolana* species. Successful fruit set has been achieved in many interspecific crosses of *Nolana* at UNH, but researchers have been unable to germinate seed from many of these fruits and make definitive designations of compatibility.

While our attempts to germinate seed of *Nolana* species and hybrids at UNH have been largely unsuccessful, seed of commercially available *N. paradoxa* var. 'Blue Bird'

germinates reliably under our conditions. *N. paradoxa* is one of few *Nolana* species known in cultivation and available commercially. The species was introduced from Chile to European gardens in the early 1820's. Over many years of cultivation, selection for ease of germination is likely responsible for higher germination rates in seed of this species.

In this study, germination of *Nolana* seed was investigated in order to develop methods of germinating seed of *Nolana* species and hybrids with increased efficiency. Success in this study was expected to increase efficiency of the *Nolana* breeding program and to add value to studies of sexual compatibility by allowing compatibility between species to be verified by hybrid seed germination.

Detailed studies of germination of *Nolana* in its natural environment have not been conducted. *Nolana* seed germinate during the humid winter months (June - September) if there is enough moisture from isolated pockets of fog to support growth of vegetation in the *lomas* (Freyre, personal communication). El Niño events occur approximately every seven to ten years, and during these times, unusually heavy fog and rainfall are experienced in the Peruvian and Atacama deserts. Dense germination of *Nolana* seedlings has been documented during these years (Dillon and Rundel, 1989). While it has been noted that increased moisture and warm temperatures associated with El Niño events increase *Nolana* seed germination rates, precise environmental conditions required for initiation of germination have not been identified as environmental data are not well documented in the isolated regions where *Nolana* are found. Additionally, the time period or the conditions to which *Nolana* seeds are exposed in the months and years prior to germination are not known. For these reasons, it is difficult for us to develop

experimental treatments that will mimic or substitute natural environmental conditions to effectively induce *Nolana* seed germination under greenhouse conditions. Rather, our studies focus on understanding the physical mechanisms of *Nolana* seed germination and investigating a variety of germination-inducing treatments commonly used in other related genera.

Study of seed germination in *Nolana* must begin with an understanding of the genus's unique fruit structure. The fruit structure of *Nolana* is a unique derived character in the Solanaceae (Knapp, 2002). *Nolana* fruit are comprised of a variable number of individual fruit segments called mericarps. Depending on the species, *Nolana* fruits reportedly consist of two to 30 individual mericarps (Tago-Nakazawa and Dillon, 1999). These highly sclerified fruit segments separate from the maternal receptacle upon ripening. Mericarps may be unilocellate or plurilocellate, reportedly containing one to seven individual seeds each (Tago-Nakazawa and Dillon, 1999). Reported mericarp and seed counts are based on observations of field-collected open pollinated fruits. Each seed within a mericarp has an associated funicular germination plug which is displaced upon germination providing a canal through which the seedling exits the mericarp (Bondeson, 1986).

The mericarp structure poses unique obstacles to the study of *Nolana* seed germination. The mericarp body is highly sclerified and difficult to dissect. *Nolana* seeds remain sealed within the mericarp until germination. It is not possible to know the number of seeds that exist within a mericarp by examining its outer surface. In some species, it is possible to count the number of funicular germination plugs on the mericarp surface, but this does not necessarily reflect the number of viable seeds within. For this

reason, we have been unable to calculate true germination rates of *Nolana* seed, but can calculate rates based only on germination per mericarp sown rather than per seed sown. Cutting or cracking a mericarp open is difficult and destructive. We have been unable to excise intact seeds from within mericarps without inflicting fatal damage. Seeds often become so mangled during manual opening of the mericarp that individual seeds cannot be distinguished and seed counts cannot be made.

Inability to excise intact seeds has also prevented viability testing using enzyme-reactive stains such as tetrazolium. Viability testing is a valuable tool in assessment of germination rates as it allows determination of germination potentials. In our studies, we have not known whether seed germination failure has been due to active dormancies or due to non-viability of the seed.

Over several years of working with *Nolana*, we have found that seeds will germinate sporadically when sown and maintained under normal greenhouse conditions for extended periods of time (up to one year), although rates of germination remain low, often below 0.1%. Observations of delayed germination suggest that while viable seeds are present, germination barriers prevent timely and reliable germination. A goal of our study was to determine whether failure of germination is due to physical germination barriers, physiological dormancy mechanisms, low numbers of viable seed, or a combination of factors. Studies reported here are preliminary experiments designed to narrow our field of focus. In many cases, due to limited supplies of mericarps, we have sacrificed replication in order to explore a wider range of germination treatments. The goal of these experiments was to gain insight into *Nolana* seed germination and identify

germination treatments which show potential and warrant additional detailed investigation.

Our study of *Nolana* seed germination began with exploration of physical characteristics of mericarps. We have used scanning electron microscopy (SEM) to examine surface characteristics and track imbibition (water uptake) using an inert dye. A series of experiments were then conducted to explore the effects of a range of chemical and environmental treatments on *Nolana* seed germination. Finally, we documented seed counts using x-ray analysis of intact mericarps and compared them to observed germination rates.

Materials and Methods

Mericarp sources

Mericarps used in this series of experiments were produced by manual hybridization on plants grown at UNH. Exceptions include wild-collected mericarps of *N. paradoxa* collected in the Atacama desert, Chile in 2005 by Dr. Michael Dillon, and mericarps of the commercial variety *N. paradoxa* var. 'Blue Bird' purchased from J.L. Hudson, Seedsman (2004). Collection information for parent accessions used in production of mericarps is listed (Table 3.1).

Soaking and scanning electron microscopy

Mericarps from eight *Nolana* species (*N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa,* wild-collected *N. paradoxa, N. plicata,* and *N. rupicola*) and one cultivated *Nolana* variety (*N. paradoxa* var. 'Blue Bird') were used. Treatments 1, 2, and 3 below were applied to mericarps of each species prior to examination by scanning

electron microscopy (SEM) or sowing for evaluation of germination rates. Mericarps of wild-collected *N. paradoxa* and *N. paradoxa* var. 'Blue Bird' were subjected to an additional four treatments (treatments 4-7 below). Except when noted, all treatments were applied on mericarps placed in 2.5 cm diameter glass vessels. Treatments were applied on 11 to 20 mericarps per species depending on availability.

Treatments include: (1) *Control*: no pretreatment; (2) *24 hour soak, then dry*: mericarps were soaked for 24 h in distilled water with gentle shaking at room temperature, followed by air drying on filter paper at room temperature for 24 h; (3) *5 day soak, then dry*: mericarps were soaked for five days in distilled water with gentle shaking at room temperature. Fresh water was replaced after two days. Mericarps were then dried at room temperature for 24 h; (4) *24 hour soak, then dry repeated twice*: mericarps were soaked and dried as in treatment 2 above, then soaked for an additional 24 h in fresh distilled water and dried as above; (5) *5 day soak, then dry, followed by 4 day soak, then dry*: mericarps were soaked for five days and dried for 24 h; followed by a second soak for four days, and dried for 24 h, as detailed above; (6) *30 minutes in H_2SO_4*: mericarps were soaked for 30 min in enough volume of concentrated sulfuric acid to cover mericarps; followed by rinsing under running water for five to ten minutes, then dried for 24 h; (7) *Boiling / ice water alternations*: mericarps were sealed in a fine mesh bag and submerged in boiling water for ten seconds, then removed and immediately submerged in ice water for ten seconds. This was repeated for six consecutive alternations of boiling water and ice water followed by a 24 h drying period.

One mericarp per species was randomly selected from each treatment for examination by scanning electron microscopy (Amray 3300FE field emission SEM with

PGT Imix-PC microanalysis system). Mericarps were viewed and digitally photographed at magnifications of 25X to 2,840X. Special attention was paid to the side of the mericarp adjacent to the maternal receptacle where germination plugs are located, and what we will refer to as the mericarp's *plug face*. Differences in physical appearance of mericarp surfaces were noted and particularly differences in surface characteristics of funicular germination plugs in response to treatments.

Remaining mericarps were sown in 10-cm azalea pots with moistened Promix-BX growing media (Premier Horticulture, Inc., Quakertown, PA) and held in a glass greenhouse for germination. Media was kept consistently moist with clear water for a period of four weeks, after which it was allowed to dry between each watering. Germination was recorded for a period of 38 weeks (February 6, 2005 – October 30, 2005). Individual seedlings were removed from pots upon germination.

Path of imbibition

Mericarps of nine *Nolana* species (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa,* wild-collected *N. paradoxa, N. plicata,* and *N. rupicola*) and the cultivated variety *N. paradoxa* var. 'Blue Bird' were used. Ten mericarps of each species were soaked in an inert blue dye solution (0.1g/L FD&C Blue #1, Sensient Colors Inc., St. Louis, MO). One mericarp per species was removed individually after time periods of 0.5 h, one h, two h, four h, eight h, 24 h, and ten days of soaking. Upon removal, mericarps were quickly rinsed with distilled water and air dried for 20 to 30 minutes. Mericarps were bisected, cutting downward through the plug face with a scalpel. Presence and location of dye solution within mericarps was recorded.

Survey of chemical and environmental treatments

Mericarps of eight *Nolana* species (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata,* and *N. rupicola*) were treated in three replicates of ten mericarps each per species. Treatments were either media-based, in which mericarps were sown in media following initial treatment; or liquid-based, in which mericarps remained soaking in treatment solutions during the germination period. Liquid treatments were carried out under ambient indoor light and temperature conditions in covered vessels. Except where noted, mericarps were soaked in a volume of 30 mL, and liquid was poured to new vials daily for aeration purposes.

Media-based treatments include: (A-1) *control:* no pre-sowing treatment; (A-2) *nitric acid:* 24 h soak in 0.1N nitric acid; (A-3) *gibberellic acid:* 24 h soak in 1,000 ppm gibberellic acid (Sigma 48880-1G-F); (A-4) *sulfuric acid:* 30 min soak in a volume of concentrated sulfuric acid covering mericarps followed by five min rinse under running tap water; (A-5) *temperature fluctuation:* mericarps sown and incubated in a growth chamber at 30°C/18°C with 12 h daylength for duration of germination period. Liquid-based treatments include: (B-1) *control:* distilled water; (B-2) *ethephon 10 mM:* (Florel, Monterey Chemical); (B-3) *ethephon 1 uM*; (B-4) *potassium nitrate 2%.*

Following treatment (media treatments, except A-5) or after 26 days of soaking (liquid treatments), mericarps were sown in 10-cm plastic pots with Fafard germination mix (Conrad-Fafard, Inc., Agawam, MA). Media was kept moist with clear water. Germination was recorded at least once weekly for 24 weeks (July 19, 2005 – January 4, 2006).

Total germination of all species combined and that of each species individually were compared with Chi-square goodness of fit test. Within each species or in the combined data, expected germination rates were calculated as the combined number of seedlings germinated in all treatments divided by the number of treatments (Table 3.2). Expected values assume no treatment effects. Germination of media-based and liquid-based treatments were analyzed independently of each other.

Natural aging

Mericarps used in this study consist of mericarps that had been produced for previous studies as well as freshly produced mericarps. Mericarps from previous studies comprise the six month, 20 month, and 24 month storage treatments. Mericarps were stored in a desiccated environment at room temperature for the duration of respective storage periods.

Mericarps of identical genetic parentage to those comprising the six month, 20 month, and 24 month storage treatments were produced by manual hybridization in the weeks preceding the start of this experiment and make up the zero month treatments. Parentages of all mericarps are listed in Appendix A. Mericarps in the zero month storage treatments were stored for up to 36 days prior to sowing depending on maturation dates of individual fruits.

Mericarps were sown in five replicates of three mericarps per family per storage treatment in D806R plastic insert trays (Griffin Greenhouse & Nursery Supplies, Tewksbury, MA) with Fafard Germination mix. Media was kept moist with clear water. Flats were held under a canopy of Reemay fabric to maintain humidity and to reduce

incidence of insect infestation. Germination was recorded weekly for 28 weeks (October 14, 2005 – April 28, 2006).

Artificially accelerated aging

Mericarps of two species, *N. aticoana* and *N. rupicola*, were produced by manual intraspecific hybridization for use in this study and were stored in a desiccated chamber at room temperature until time of treatment. Duration of pre-treatment storage period ranged from two to 18 weeks depending on maturation dates of individual fruits.

Forty-five mericarps per species were included in each pre-sowing treatment. Treatments included 13 warm storage (35°C) durations (zero to 12 weeks storage) and two levels of storage humidity (desiccated or 75% relative humidity). Atmosphere of 75% relative humidity (RH) was maintained in a nine liter air-tight plastic container by including a 0.5 liter volume of sodium chloride saturated salt solution (Greenspan, 1977). A desiccated atmosphere was maintained in a second nine liter container using activated silica gel (Eagle Chemical Co., Inc., Mobile, AL). Containers were incubated at constant 35°C with 24 h darkness. Mericarps were stored in paper coin envelopes within the plastic containers during treatment.

Treatments were initiated at staggered intervals to maintain a schedule in which all pre-sowing treatments concluded and mericarps were sown on the same date. Mericarps were sown in randomized replicates with nine replicates per treatment and five mericarps per replicate in D806R plastic insert trays with Fafard germination mix. Flats were held in a glass greenhouse under a canopy of Reemay. Media was kept moist with clear water. Germination was recorded at least once per week for 16 weeks (January 10, 2006 – May 2, 2006). Germination in response to warm-humid treatment was compared

to that of warm-dry treatment by analysis of variance (ANOVA) (Systat 10, SPSS Inc, 2000).

Imbibition rates

Mericarps of eight species (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata,* and *N. rupicola*) were produced by artificial hybridization for use in this study. One-hundred mericarps per species were submerged in 30 mL dye solution (0.1g/L FD&C Blue #1, Sensient Colors Inc., St. Louis, MO) for up to 14 days. Solution was replaced every one to two days to provide aeration.

Five mericarps per species were removed at random from the dye solution daily. Mericarps were quickly rinsed with distilled water, patted dry, and air dried for 20 to 30 minutes. Mericarps were bisected by cutting through the funicular plug face. Each visible seed was observed for presence of imbibed dye solution. Number of mericarps with one or more seeds exhibiting dye imbibition was recorded for each species daily. Observations were made for 14 days until all species exhibited imbibition in all five mericarps.

Imbibition rate indices were calculated for each species. Indices were calculated as the average number of mericarps exhibiting imbibition per day over 14 days of observation. Imbibition rate indices were compared to increases in germination in response to the 24 h gibberellic acid soak performed in Experiment 3 over the control treatment. Values were compared using Spearman's rank order correlation (Systat 10, SPSS Inc, 2000). Species (*N. ivaniana* and *N. laxa*) which failed to germinate in both control and gibberellic acid treatments were excluded from correlation analysis.

Seed counts by x-ray analysis

Mericarps of eight species (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata,* and *N. rupicola*) and 27 interspecific hybrid families were produced by manual hybridization. Details of hybrid parentage are reported in Appendix B. Mericarps were x-rayed using the Faxitron MX20 Digital X-ray at Ohio State University's Ornamental Plant Germplasm Center. Twelve to 286 mericarps were x-rayed per species or hybrid family. Digital images of each x-ray were recorded. Designations of presumed full seeds and of cavities appearing empty or appearing to contain abnormally formed seed in each mericarp were made based on differences in contrast within recorded images. Mericarps (excluding those of hybrid families) were arranged on Scotch tape for x-raying and were kept on the tape so that each mericarp could later be identified from the recorded images.

Species mericarps were sown as described below in Experiment 8. Germination from individual mericarps was compared to presumed seed counts made by analysis of x-ray images. Hybrid mericarps were sown August 2006 in 128-plug trays with a 2:1 peat:perlite propagation medium at a distribution of one mericarp per cell. Mericarps were germinated under natural day lengths in a glass greenhouse with an average temperature of 22°C and relative humidity of 73%. Number of seedlings germinating from each mericarp was recorded.

Modified gibberellic acid and warm temperature treatments

Mericarps used in this study were selected from the pool of mericarps appearing to contain one or more full seeds as determined by x-ray analysis. Mericarps of *N. laxa* were randomly selected from the total pool of mericarps used in x-ray analysis, as

differences in contrast in mericarps could not be distinguished as described above. Numbers of mericarps included in each treatment ranged from nine to 20 mericarps. Mericarps were stored in a desiccated chamber at room temperature until treatment initiation.

Treatments include (1) *no treatment*; (2) *GA_3 soak*: mericarps were placed plug face down on gauze saturated with 1,000 ppm gibberellic acid (GA_3) for species-specific durations (three to ten days as described below) at room temperature with 12 h daylength; the gauze was re-saturated with GA_3 as needed; (3) *warm temperature treatment sown in media*: mericarps were sown into individual cells of 196-plug trays with Fafard germination mix and incubated at 35°C, 75%RH for seven weeks; moisture was maintained with clear water; (4) *warm temperature treatment in envelopes*: mericarps were held in paper coin envelopes and incubated at 35°C, 75%RH for seven weeks; humidity was maintained with a saturated salt solution; (5) *GA_3 soak followed by warm temperature treatment*: mericarps were placed plug face down on gauze saturated with 1000 ppm GA_3 for species-specific durations as described below, then sown and incubated at 35°C, 75%RH for seven weeks; moisture was maintained with clear water; (6) *warm temperature treatment in envelopes followed by GA_3 soak*: mericarps were held in paper coin envelopes and incubated at 35°C, 75%RH for seven weeks, then soaked with 1,000 ppm GA_3 for species-specific durations as described below; (7) *GA_3 soak with high temperature (50°C) treatment*: mericarps were placed plug face down on gauze saturated with 1,000 ppm GA_3 for four days at 50°C with 12 hour daylength; gauze was re-saturated with GA_3 as needed; (8) *two days of high temperature treatment*: mericarps held in coin envelopes and incubated at 50°C, 24 h darkness in a desiccated chamber for

two days; (9) *four days of high temperature treatment*: mericarps held in coin envelopes and incubated at 50°C, 24 h darkness in a desiccated chamber for four days and (10) *six days of high temperature treatment*: mericarps held in coin envelopes and incubated at 50°C 24 h darkness in a desiccated chamber for six days.

Target soaking durations were determined for each species to ensure imbibition of gibberellic acid solution while preventing possible toxic effects of prolonged soaking. Number of days required for imbibition to occur was determined in Experiment 6. Here, we added two days to these values to provide ample time for complete uptake of GA_3.

Treatments were initiated at staggered intervals with all treatments concluding on the same day. Mericarps of treatments 1, 2, 4, 6-10 were sown randomly and singly into individual cells of 196-plug trays with Fafard germination mix and covered with Reemay. Mericarps of treatments 3 and 5 were sown in this manner during treatment, and were moved to the greenhouse at conclusion of treatment. Media was kept moist with clear water. Germination was recorded weekly for 21 weeks (June 2, 2006 – October 27, 2006).

Cold stratification

Mericarps of *N. humifusa* and *N. rupicola* were produced by manual hybridization and were stored at room temperature in a desiccated chamber for two to five months prior to treatment initiation. Treatments included (1) *control:* no cold treatment; (2) *two weeks cold stratification*; (3) *four weeks cold stratification*; (4) *eight weeks cold stratification* and (5) *twelve weeks cold stratification.* Forty mericarps were sown per species per treatment.

In preparation for cold stratification, mericarps were sown at a rate of one mericarp per cell in 196-plug trays with Fafard germination mix and moistened with clear water. Cold stratification was then carried out at 12°C with 12 h days for two to 12 weeks as described above. Treatment initiation was staggered allowing all treatments to be moved to ambient greenhouse conditions on a single day. Germination was recorded weekly for 16 weeks (April 2, 2006 – July 24, 2006).

Sterile culture of immature seed

Mericarps of three species (*N. humifusa, N. plicata*, and *N. rupicola*) were produced by manual intraspecific hybridization. Ripe harvest is designated as the stage at which mericarps are dark in color and loosened from the receptacle. Hybridization dates were staggered allowing mericarps of five fruits from each of five maturity levels to be sown in culture on a single date (August 31, 2006). Maturity levels included (1) one week past ripe harvest (fruit was harvested and mericarps were then stored at room temperature); (2) ripe harvest; (3) one week prior to ripe harvest; (4) two weeks prior to ripe harvest; (5) four weeks prior to ripe harvest.

Mericarps were surface sterilized with 30% bleach solution for ten minutes followed by three rinses in sterile water. Funicular germination plugs are not fully formed at this stage, therefore non-ripe mericarps were left attached to the receptacle during sterilization to prevent direct exposure of seeds to sterilization solution. After sterilization, mericarps were separated from the receptacle and sown on 40 mL sterile embryo rescue medium (Appendix C) in Magenta GA-7 vessels (Magenta Corp., Chicago, IL). Mericarps were sown at a rate of four to five mericarps per vessel and

were incubated at room temperature with 12 h day length for 16 weeks (August 29, 2006 – December 19, 2006).

Results and Discussion

This series of experiments explores a range of factors associated with seed germination in *Nolana.* These experiments build on one another as we investigate mechanisms of germination and causes of germination failure. These experiments are designed to provide background knowledge required for more in-depth studies of factors contributing to *Nolana* seed germination and to narrow the field of focus for future studies.

Soaking and scanning electron microscopy

Nolana mericarps germinate in their natural desert environment during yearly periods of high humidity, and at particularly high rates during el Niño years when rainfall occurs in areas of the hyper-arid Peruvian and Atacama deserts. Based on these observations of conditions supporting natural germination of *Nolana*, we explored the effects of exposure to water on physical attributes of mericarps and on seed germination. Mericarps were soaked for short periods (24 h), longer periods (five days) or cycles of soaking and drying to simulate natural patterns of exposure to moisture. We hypothesized that germination failure was due to a physical barrier, with the funicular germination plug blocking water entry to the seed or blocking growth and emergence of the seedling. In theory, cycles of soaking and drying would facilitate germination by loosening the funicular germination plug through hydration and drying of the cells or matrix associated with the plug.

Following soaking treatments, mericarps were viewed by scanning electron microscopy (SEM) to identify changes in mericarp surface attributes, with special attention paid to the funicular germination plugs and surrounding area. We looked for evidence of loosening of the funicular plugs or other changes suggestive of increased preparedness for germination.

Due to the small scale of this study with only one mericarp per species analyzed by SEM following each treatment, our ability to conclusively identify physical changes resulting from soaking treatments was limited. In instances where physical differences were apparent, differences involved integrity of a coating of unknown composition which covers the mericarp surface. Treatment of mericarps with concentrated sulfuric acid resulted in degradation of this coating exposing a porous mericarp body. Meanwhile, in non-soaked mericarps or those soaked in water, the surface coating remained largely intact (Figure 3.1). Less striking differences were apparent in the integrity of the surface coating between non-acid treated mericarps. Integrity of the coating varied by species from largely complete and fairly smooth in *N. paradoxa* to quite porous in *N. humifusa* (Figure 3.1 *a, e*). These differences, however, do not appear to be correlated to soaking treatments. Variation in coating characteristics between mericarps of *N. paradoxa* from five treatments is illustrated in Figure 3.2. Coating appears largely complete and smooth on non-soaked mericarps, those exposed to two cycles of 24 h soaking, and those exposed to two cycles of four to five days of soaking. Coating on mericarps exposed to 24 h or five days of soaking appears incomplete and porous funicular plug tissue is exposed. These differences are likely due to differences in growth conditions during fruit

development rather than soaking treatments as the differences are not consistently correlated to soaking durations or cycles.

A morphological feature of special interest was identified in mericarps of six species. A small ring (20 – 40 μM in diameter) of coiled tracheid cells was found located in the center of the funicular germination plug. These coiled cells presumably make up the seed's funicle. This feature was visible in only a small number of mericarps, presumably in others it remained concealed beneath the surface coating (Figure 3.3). We hypothesized that this tube of cells may function as a direct path of water uptake into enclosed seeds. If this were found to be the case, presence of a physical barrier to water uptake could be eliminated as a possible cause of germination failure. We explored this possibility in Experiment 2.

Effects of soaking treatments on germination were evaluated by sowing mericarps from each treatment and measuring germination rates (Table 3.2). As expected, mericarps of the cultivated variety *N. paradoxa* var. 'Blue Bird' germinated at the highest rate (0.49 seedlings per mericarp).

Treatment of boiling/ice water alternation was applied only to mericarps of *N. paradoxa* var. 'Blue Bird' and *N. paradoxa*. This treatment appears to be detrimental to germination in these species, with no germination occurring in either species. Low germination rates prevent detection of significant trends in response to other soaking treatments, but no treatment appears to have substantially increased germination in any species.

Path of imbibition

Analysis of soaking treatments in Experiment 1 did not contribute significantly to our understanding of *Nolana* germination requirements. However, identification of a tube of tracheid cells in the center of the germination plug provided evidence that a physical barrier to imbibition (water uptake to the seed) may not be a factor contributing to germination failure. A common physical barrier to germination in other species is presence of an impermeable seed coat which prevents imbibition. In these cases, physical scarification is often required to break down or open the seed coat to facilitate water entry. If the tube of cells seen in *Nolana* germination plugs does function as a direct path of water uptake to seed without requiring scarification, focus of our research could be shifted to exploring factors associated with other types of dormancy.

Our goal was to determine whether imbibition occurs in *Nolana* without scarification treatments and if the funicle functions as a conduit for water entry. Mericarps were soaked in an inert blue dye solution and were dissected after a time period of 30 min to ten days of soaking. Location of dye solution within the dissected mericarps was noted. We found that imbibition occurs in all studied species without scarification and that the funicle does in fact function as the path of water uptake.

The process of imbibition was most clearly visible in mericarps of the cultivated variety *N. paradoxa* 'Blue Bird' and in the species *N. paradoxa*. Mericarp tissue of these two species was much softer than that of the other studied species, allowing for cleaner cuts during dissection and limited damage to the internal mericarp structure. Imbibition occurred quickly in these samples with substantial hydration of the seed coat after only 24 h. Figure 3.4 illustrates the path of dye uptake as observed in dissected mericarps of

N. paradoxa. By observing mericarps at each time interval, we were able to visualize the progression of imbibition. After 30 min of soaking, the dye was observed within the center of the funicular germination plug and beginning to hydrate the tissue surrounding the seed's hilum. After 4 h, the dye had hydrated a pad of tissue on the underside of the germination plug adjacent to the radicle end of the seed. In later time periods, the dye was seen spreading up the sides of the germination plug and loosening it, evidently in preparation for germination. In mericarps that had not undergone soaking, the germination plug appeared tightly sealed within the germination canal. After soaking, germination plugs appeared notably loosened. After 24 h of soaking the dye was seen as hydrating the entire seed coat. Throughout this process, the funicle was the only identified point of entry for the dye solution. After ten days of soaking, the dye was seen as saturating the outer layers of the mericarp body, but not penetrating the seed from any spot other than the funicle. Evidence of similar pattern of hydration was seen in all studied species.

While species-specific differences in the time it takes for imbibition to occur can not be quantified from the data produced in this experiment, the fact that differences exist was clearly demonstrated. Evidence of imbibition was seen in mericarps of *N. paradoxa* after only 30 min of soaking while signs of dye uptake into mericarps of *N. plicata* and *N. rupicola* were seen only after several days of soaking. Interestingly, imbibition occurred most quickly in those species which have been most successful in germination in past experiments while those that have been less successful in germination took longer to imbibe the solution. This finding suggests that while imbibition occurs in all studied species without requiring scarification, differences in rates of imbibition may play a role

in germinability. Those species which are slower to imbibe may require longer duration of exposure to water or exposure to a more saturated medium than that which we have provided in previous germination attempts.

Survey of chemical and environmental treatments

Successful imbibition of *Nolana* seed without scarification suggests that the seeds' failure to germinate is due to a physiological dormancy rather than a physical barrier. Seeds affected by physiological dormancy are able to imbibe water, but cannot germinate until certain environmental requirements are met (Baskin and Baskin, 1998). Common environmental requirements include exposure to light or darkness or to high, low, or alternating temperatures. In many cases, physiological dormancies may be broken artificially by exposure to one or more of a variety of chemicals (Egley and Duke, 1985; Baskin and Baskin, 1998). Common dormancy breaking chemicals include potassium nitrate, thiourea, kinetin, ethylene, and gibberellins (Baskin and Baskin, 1998).

We explored the efficacy of a variety of chemical and environmental treatments in increasing *Nolana* seed germination rates. Treatments were selected from those cited as having positive effects on germination in other Solanaceae and/or desert species. Alternating temperatures of 18°C/30°C have, alone or in combination with other treatments, successfully induced germination in many species within the Solanaceae as have treatments with 2% potassium nitrate or 0.1N nitric acid (Sudiatso and Wilson., 1974; Ellis, et al., 1985; Bithell, et al., 2002). Gibberellic acid is commonly used to overcome dormancy in many genera, including many in the Solanaceae (Baskin and Baskin, 1998; Bithell, et al., 2002; Pingle and Dnyansagar, 1979; Suchorska and Ruminska, 1980). Exposure to ethylene in the form of ethephon at various rates has

proven successful in overcoming dormancy in desert species (Khan, et al., 2003). The objective of this study was to identify treatments which have a positive effect on germination rates and may be modified in successive studies to further increase treatment's germination-inducing effects.

Table 3.3 summarizes germination results. Treatments are separated into liquid-based (those in which mericarps remained soaking in liquid treatment for a prolonged period) and media-based (those in which mericarps were sown in media following treatment). Liquid-based treatments were largely unsuccessful in inducing germination with an overall germination rate of 0.08 seedlings per mericarp. Germination in response to liquid treatments occurred in five out of eight species (*N. adansonii, N. aticoana, N. elegans, N. laxa*, and *N. plicata*), however only one species, *N. aticoana*, exhibited a notably high response to any treatment with a germination rate of 0.23 seedlings per mericarp in response to soaking in 10 mM ethephon compared to 0.00 in all other liquid-based treatments. Other species did not show a positive response to this treatment. Additionally, germination of *N. aticoana* in response to this treatment did not surpass that of *N. aticoana* sown under normal greenhouse conditions (treatment A-1). We therefore do not find ethephon soaking to be a promising treatment worthy of further investigation.

Chi-square goodness of fit analysis indicates a significant difference in germination of *Nolana* between media-based treatments when considering cumulative germination among all species (χ^2 $p<0.001$). Germination from mericarps treated with gibberellic acid was notably higher than that from all other media-based treatments (Table 3.3). In total, 40 seedlings germinated from gibberellic acid soaked mericarps while germination in other treatments ranged from just one to 13 seedlings. Chi-square

goodness of fit analyses of individual species revealed significant treatment effects in four of the eight species (*N. aticoana*, *N. elegans*, *N. humifusa*, and *N. plicata*) (Table 3.2). A trend in treatment response was observed across species with germination in response to gibberellic acid being notably higher than the expected germination rate based on chi-square calculations and higher than germination in all other treatments in *N. elegans*, *N. humifusa*, and *N. plicata*. Of the remaining species, two (*N. ivaniana* and *N. laxa*) failed to germinate in all treatments, two exhibited statistically equal germination in response to each treatment (*N. adansonii* and *N. rupicola*), and one (*N. aticoana*) germinated equally well in all treatments except sulfuric acid. Differences in species' responses to gibberellic acid may be indicative of varying levels of dormancy with species affected by deeper dormancies showing little or no response to the treatment (Finch-Savage and Leubner-Metzger, 2006). Alternatively, the differences may be tied to differences in rates of imbibition between species as previously described. Twenty-four hours of exposure to gibberellic acid may not have been long enough for the hormone solution to be imbibed into seeds of all species, and longer soaking periods may be required.

Interestingly, germination in response to treatment with gibberellic acid continued for many weeks after germination in other treatments had ceased (Figure 3.5). For other treatments most germination occurred within four weeks of sowing and increased very little thereafter. Germination in response to gibberellic acid, however, increased more than three-fold over the following 20 weeks. A possible explanation for this phenomenon is that exposure to gibberellic acid initiated activation of metabolic processes required for

germination. Differences between seeds in rates of these processes or in initial readiness to germinate could account for the range of germination times.

Gibberellic acid showed great potential as a germination-inducing treatment for *Nolana*. Germination in response to gibberellic acid far exceeded that of other treatments, however final germination rate is still very low at 0.17 seedlings per mericarp. As *Nolana* mericarps presumably contain multiple seeds per mericarp, ideal germination rates could potentially be above one seedling per mericarp. Through modification of gibberellic acid treatment or implementation of treatments with similar modes of action we hope to attain acceptable rates of germination.

Natural aging

The observed increase in germination in response to treatment with gibberellic acid supports our hypothesis that low germination rates in *Nolana* are likely due to physiological dormancy. Physiological dormancy is often caused by presence of chemicals which inhibit metabolic processes responsible for readying seeds for germination. Treatment with gibberellic acid overcomes inhibitory effects of these chemicals, triggering activation of metabolic processes and, in turn, allowing for germination to occur. Alternatively, effects of chemical inhibitors may be broken down over time as seeds remain in dry storage (commonly referred to as after-ripening), allowing older seed to germinate more readily than fresh seed (Bewley, 1997). Based on our casual observations made in previous unpublished studies involving germination of *Nolana*, a relationship appears to exist between duration of pre-sowing dry storage periods and seed germinability. Mericarps which have undergone long periods of dry storage appear to germinate at higher rates than do those stored for short periods. Storage

periods ranging from several months to one year or more may positively affect *Nolana* seed germination.

To determine whether germination rates of *Nolana* seed are affected by length of pre-sowing dry storage, we compared germination of freshly produced mericarps against others which had been held in storage for six, 20 or 24 months. Unfortunately it was not possible to obtain sets of mericarps with identical parentage stored for the different time periods. Intrinsic differences may exist in germinability between mericarps of differing parentage, thus germination cannot be confidently compared between all sets of mericarps. Statistical comparisons may be made only between aged mericarps and freshly produced replicates of identical parentage.

Germination results are summarized in Table 3.4. All germination occurred within three weeks of sowing at an overall rate of 0.10 seedlings per mericarp. Comparison of germination from six month aged mericarps to that of fresh replicates reveals little difference in overall germination rates, with ten seedlings germinating from aged mericarps and six germinating from fresh mericarps. Differences are apparent, however, in distribution of germination between species within the two groups. Ten seedlings germinating from six month aged mericarps belong to four species (*N. adansonii, N. aticoana, N. elegans,* and *N. plicata*), whereas five out of six seedlings germinating from the fresh replicates belong to a single species (*N. adansonii*). Although low germination rates prevent statistical analysis of these results we conclude that germination was more successful with mericarps which had been held storage for six months since it was effective for several species.

Germination in 24-month aged mericarps was highly successful with 116 seedlings germinating from 210 sown mericarps. Germination of corresponding fresh replicates was minimal with only one seedling germinating from 210 sown mericarps. Germination of 24-month aged mericarps was well distributed with germination occurring in four out of five hybrid families. In this case, germination of stored seed was far more successful than that of fresh seed.

No germination occurred in the twenty month aged mericarps or in corresponding fresh replicates. Lack of germination from these fresh mericarps is not unexpected considering low germination rates from other fresh mericarps in this study. However, lack of germination in mericarps stored for 20 months is unexpected. This failure of germination may be more closely related to innate germinability of the species involved rather than effects of storage. The four species included in the 20 month storage treatment exhibited similarly low germination rates in all other treatments as well. Only one seedling germinated out of 600 total mericarps sown in aged and fresh replicates for these four species (*N. humifusa, N. ivaniana, N. laxa,* and *N. rupicola*). Perhaps longer storage periods are required for these species.

Results of this study do not provide conclusive evidence of effects of aging on germination of *Nolana* seed. However, results suggest that aging may be an effective means of inducing germination in some *Nolana*. Interestingly, germination was most successful in mericarps of interspecific hybrid families. However, based on these results, we cannot conclude that genetic parentage is associated with the observed germination success.

Artificially accelerated aging

The physiological changes which seeds undergo during prolonged storage may be accelerated under modified storage conditions. Exposure to warm temperatures increases rates of metabolic processes within the seed, speeding effects of the natural aging process (Finch-Savage and Leubner-Metzger, 2006; Bewley, 1997). Warm temperature storage has been shown to effectively induce germination in many species exhibiting endogenous physiological dormancies (Egley and Duke, 1985; Kucera et al., 2005). Endogenous physiological dormancy may also be the cause of low germination in *Nolana*. To evaluate the effectiveness of artificial aging on *Nolana* germination, we selected two species (*N. aticoana* and *N. rupicola*) to be exposed to warm temperature treatments. By exposing mericarps to warm temperatures for a range of durations and at differing humidity levels, we aimed to identify a treatment which would induce germination at acceptable rates in a relatively short period of time, eliminating the need to store mericarps for many months prior to sowing. In the previous experiment *N. aticoana* exhibited a positive response to natural aging while *N. rupicola* showed no response.

N. rupicola failed to germinate in response to all treatments in this study. *N. rupicola* has also exhibited very low germination in all our germination studies, suggesting that dormancy may be deeper in this species than in other studied species. Therefore germination requirements differ between species and species-specific modifications of treatments may be neccessary.

N. aticoana achieved an overall germination rate of 0.09 seedlings per mericarp. Germination results are illustrated in Figure 3.6. Although differences between treatments are evident, low germination rates prevent statistical analysis of treatment

effects in this study. Mericarps exposed to warm temperature storage achieved an overall germination rate of 0.10 seedlings per mericarp compared to no germination in the control treatment (zero weeks of warm temperature exposure). Warm-humid storage was significantly more effective in induction of germination than was warm-dry storatge, with warm-humid storage achieving an overall germination rate of 0.19 seedlings per mericarp and warm-dry storage achieving a rate of only 0.03 seedlings per mericarp (ANOVA $p<0.05$). Typically, metabolic rates are higher in imbibed seeds such as those exposed to humid environments, making these seeds more receptive to environmental germination cues such as elevated temperatures (Bewley, 1997).

Minimal differences in germination rates were seen between durations of warm-dry storage. No more than four seedlings germinated from any warm-dry treatment duration. Germination in warm-humid treatments ranged between duration periods from two to 16 seedlings. Treatment durations of three to six and eight to 12 weeks appear to be equally effective in inducing germination. A peak in germination occurred in the seven week warm-humid treatment with a germination rate of 0.40 seedlings per mericarp compared to an average of 0.16 seedlings per mericarps in other warm-humid treatments. A germination rate of this magnitude is far greater than rates achieved in previous experiments. However, this treatment was shown to be successful with only a single species. *N. rupicola* showed no response to this treatment and effect on other species was not explored. This study does, however, provide strong evidence that warm-humid treatment may be an effective means of inducing germination in some species of *Nolana* and should be explored further. Differences in germinability between species may require variation in treatment duration or temperature.

Imbibition rates

Previously, we demonstrated that soaking mericarps in gibberellic acid for 24 h prior to sowing was effective in increasing germination rates in some species of *Nolana*. However, the increase in germination varied between species. We hypothesized that 24 h of soaking may not be long enough for imbibition of the chemical in some species. Seeds of species which are slow to imbibe may have received little or no exposure to the chemical. The objective of this experiment is to quantify the number of days of soaking required for imbibition in mericarps of different *Nolana* species. This data will allow us to modify soaking times in future studies to ensure imbibition.

In this study, a mericarp was considered to have undergone imbibition when dye was visible on at least one of the mericarp's enclosed seeds. Only those seeds visible upon bisection with a single cut through the mericarp plug face were considered. Consideration was not given to the number of seeds in a mericarp or to the percent of seeds within a single mericarp having undergone imbibition. The number of mericarps having undergone imbibition was recorded daily for each species and is reported in Table 3.5.

Differences between species in the rate of imbibition were evident. *N. humifusa* and *N. plicata* exhibited the most rapid rate of imbibition with all five examined mericarps showing imbibition after one day of soaking. The process was similarly rapid in *N. aticoana* with four out of five mericarps imbibed after one day and five out of five imbibed after two days. Contrarily, *N. rupicola*, *N. adansonii,* and *N. elegans* exhibited the slowest rates of imbibition with five out of five mericarps showing imbibition only after 12, ten, and eight days of soaking, respectively.

Calculation of the number of days of soaking required for five out of five mericarps to show imbibition may not be a fully accurate representation of a species' rate of imbibition because species exhibited different percentages of imbibed mericarps in the days prior to the day on which five out five mericarps were imbibed. A more representative value of the species' imbibition rates may be calculated as the average number of mericarps exhibiting imbibition daily over the number of days of soaking required for all eight species to exhibit imbibition in five out of five mericarps (14 days). This calculated 'imbibition index' is reported in Table 3.5 and illustrates the relative rate of imbibition between the eight studied species. The highest imbibition index belongs to *N. plicata* indicating that this species imbibed most quickly and reliably. *N. adansonii* had the lowest imbibition index indicating that this species was slower to imbibe than others.

Identification of species-specific differences in imbibition rates allow for more precise studies of effects of soaking treatments on germination of *Nolana* seed. With this data, mericarps may be subjected to species-specific soaking durations designed to ensure imbibition of the soaking solution. For example, it is particularly useful for soaking mericarps in solutions of gibberellic acid to stimulate germination. As shown previously, soaking mericarps in a 1000 ppm solution of gibberellic acid for a period of 24 h positively affected germination rates in five out of eight studied species. The magnitude of the increase, however, remained insufficient for practical germination purposes. It is therefore desirable to modify the gibberellic acid treatment to optimize its effectiveness in promoting germination in *Nolana*. The key to modification of this treatment may lie in the differences between the identified species-specific rates of imbibition.

A comparison of species-specific effects of 24 h gibberellic acid soak reported in Table 3.3 to species-specific imbibition rates reported in Table 3.5 support our hypothesis that some species may require longer soaking periods to fully benefit from gibberellic acid treatment. As hypothesized, species that show the strongest positive response to 24 h gibberellic acid soak (*N. humifusa* and *N. plicata*) require only one day of soaking for imbibition to occur. The soaking duration of 24 h was presumably sufficient for gibberellic acid to reach the seed and stimulate germination. Meanwhile, species that require longer soaking durations for imbibition to occur exhibited a reduced response to the gibberellic acid treatment, likely because 24 h of soaking was insufficient for the gibberellic acid to be imbibed to a majority of the seeds. A significant correlation exists between species' degree of response to gibberellic acid treatment, as measured by increase in germination of gibberellic acid treated seed over control seed, and species' imbibition indices (Spearman Rank Order Correlation $r_s = 0.638$, $p \leq 0.05$). Results suggest that a stronger response to gibberellic acid soak in all species may be achieved with the implementation of longer soaking times.

Seed counts by x-ray analysis

Throughout our studies, we have expected that germination rates in excess of one seedling per mericarp are attainable in *Nolana* based on theoretical presence of multiple seeds per mericarp. We have been unable, however, to measure actual numbers of seeds within the stony fruit segments due to our inability to dissect mericarps without inflicting fatal damage to the enclosed seeds. In many cases, damage incurred during opening of mericarps has been so severe that individual seeds cannot be distinguished. Because of this, we have been unable to make accurate estimates of germination potentials and have

been unable to accurately evaluate effectiveness of germination treatments. Low germination rates may be due, in part, to low seed set or low seed viability. To evaluate impact of these factors, we must determine seed counts for individual mericarps.

Non-destructive x-ray analysis has been successfully used to evaluate seed set and seed quality in many species including *Petunia*, *Oenothera*, *Talinum*, and several grains (Craviotto, et al., 2004; Steive and Tay, 2006). Previously reported use of x-ray analysis, however, has been limited to individual seeds. Efficacy of viewing seeds within intact fruiting structures, such as mericarps of *Nolana*, has not been evaluated before, as far as we are aware. We conducted x-ray analyses on mericarps of several *Nolana* species and interspecific hybrids at the University of Ohio's Ornamental Plant Germplasm Center and it was effective for visualizing *Nolana* seeds and determining seed counts.

Based on differences in contrast within the x-ray images, estimates were made of the number of fully formed seeds and the number of abnormal seeds or empty seed cavities within mericarps. Figure 3.7 shows a sample x-ray image of *Nolana* mericarps. The designation of *full seed* was made when image contrast within a mericarp cavity was bright, indicating the presence of dense tissue. The designation of *empty cavity* was made when contrast of a mericarp cavity was dark indicating vacant space, and that of *abnormal seed* was given when a mericarp cavity appeared to contain some dense tissue but it did not fill the cavity (Figure 3.7). Several mericarps were dissected to verify accuracy of designations based on contrast.

Table 3.6 summarizes results of x-ray analysis. Data are not available for mericarps with seed parent *N. laxa* because the species' unique mericarp morphology inhibited our ability to detect differences in contrast as described above. X-ray analysis

showed that, as expected, mericarps were largely plurilocular with greater than one seed cavity per mericarp on average. Numbers of cavities per mericarp ranged between species from 1.06 in *N. adansonii* to 7.43 in *N. plicata*. Hybrid families exhibited a similar range with an average of 1.05 cavities per mericarp in the family *N. adansonii x N. aticoana* to 8.58 in *N. plicata x N. laxa*. Interestingly, a mix of both full seeds and empty cavities or abnormal seeds were found within mericarps of most species and hybrid families. Only one family (*N. ivaniana x N. aticoana*) resulted in mericarps with 100% cavities appearing to contain full seeds. On average, only 54% of the observed cavities within mericarps were found to contain full seeds. These results show that even in cases where funicular germination plugs on the mericarp surface can be counted, this value does not necessarily correlate to the number of seeds within.

The number of full seeds per mericarp (those that are presumed to be fully developed and viable) was compared between species and between hybrids, and differences were found to exist. Average number of full seeds per mericarp ranged from 0.86 in *N. adansonii* to 5.03 in *N. plicata*. Average seed counts in hybrid families ranged from 0 in *N. plicata* x *N. laxa* to 4.00 in *N. ivaniana* x *N. rupicola*. Six out of seven analyzed species were found to contain greater than one full seed per mericarp (*N. adansonii* is an exception with an average of 0.86 seeds per mericarp). On average, lower seed set was achieved in interspecific hybrids, but seed set of at least one seed per mericarp was observed in 19 out of 27 families. Based on seed counts, we determine that our goal of achieving germination rates in excess of one seedling per mericarp is reasonable in most cases.

We attempted to germinate seed from x-rayed mericarps. For the first time in our studies, we were able to calculate a precise germination rate based on number of seeds sown, rather than one based solely number of mericarps sown (Table 3.7). As expected based on results from our previous experiments, germination rates were low, with germination falling far below the estimated number of seeds sown. Overall, a germination rate of 0.11 seedlings per seed sown was achieved.

The number of seedlings germinating from each individual mericarp was recorded. A total of 350 mericarps germinated and in most cases, only one seedling germinated from each mericarp. In spite of the fact that many mericarps contained more than one full seed, germination of two or three seedlings occurred in only 51 (Table 3.8). Germination events within individual mericarps were most commonly separated one or two days, however longer durations of up to 79 days were also observed. Results indicate that some seeds within a single mericarp may have different germination requirements. This may be an adaptation to the harsh environment to which *Nolana* are endemic. Seed which does not germinate in response to the initial occurrence of suitable environmental conditions will remain as back up in the event the conditions change and seedlings which initially germinated die.

Modified gibberellic acid and warm temperature treatments

As discussed previously, 24 h soak in gibberellic acid prior to sowing was only moderately successful in increasing germination rates in *Nolana*. Differences in imbibition rates were identified between species and were theorized to be a factor in limited success of our attempted gibberellic acid treatment. Warm temperature treatment was successful in increasing germination in one *Nolana* species, but showed no effect on

germination in another. These treatments showed potential but not ideal results, therefore we aimed to increase their benefits by combining and modifying variations of gibberellic acid and warm temperature treatments.

Germination results are reported in Table 3.9. Low germination rates prevent statistical analysis of these data, but casual analysis does not reveal increased germination in response to any treatment. Minimal differences in germination rates exist, but species are variable in response to treatments with germination within treatments increased in some species and decreased in others.

In this study, mericarps were soaked in 1000 ppm gibberellic acid for species-specific soaking durations as calculated previously based on time required for imbibition of dye solution in each species. The effect of soaking mericarps in gibberellic acid for species-specific durations was not directly compared to effect of soaking for 24 h, however species-specific soaking durations do not appear to amplify beneficial effects of gibberellic acid observed in our earlier study. In this study, germination from mericarps soaked in gibberellic acid for species-specific durations was only minimally higher than that of the control. These results disagree with our hypothesis that longer soaking durations will increase germination by assuring exposure of seeds to gibberellic acid solution.

Combined effects of warm temperature treatment and gibberellic acid treatment at species-specific durations were explored. Seven weeks of warm treatment (35°C) followed by gibberellic acid treatment appeared to be more successful than the reciprocal gibberellic acid treatment followed by seven weeks of warm temperatures. However, due

to limited availability of seed, the second treatment was applied to only two species, limiting our ability to draw conclusions regarding treatment success.

Warm temperature treatment was previously found to successfully increase germination rates in *N. aticoana*. In this experiment we evaluate effect of this treatment on eight different species. We exposed mericarps to warm temperatures for seven weeks either stored in coin envelopes at 75% relative humidity as in the previous study, or sown in moist media. Results were variable between species. *N. aticoana* was the only species to respond best to treatment at 75% relative humidity, although increase in germination was little more than that of the control. Four species (*N. adansonii, N. humifusa, N. ivaniana*, and *N. plicata*) exhibited germination in response to warm temperature treatment while sown in media but not while stored at 75% relative humidity. Germination in these cases was only slightly higher than that of the control.

Use of high temperature treatment (50°C) does not appear to be effective in inducing germination in *Nolana*. Treatment of mericarps of *N. humifusa* with two, four, or six days of high temperature treatment prior to sowing resulted in no germination. It is not possible to ascertain whether the high temperature in fact enhanced dormancy or killed the seed. Treatment with gibberellic acid negates the dormancy-enhancing effects of high temperatures (Haber, 1965). However, treatment of mericarps of *N. humifusa* and *N. aticoana* at high temperature while soaking in gibberellic acid for four days did not show increased germination as compared to the control treatment.

Cold stratification

A common treatment for overcoming physiological seed dormancy is cold stratification. Exposure of seeds to cool temperatures for durations of several weeks to

several months has effectively induced germination in numerous species (Baskin and Baskin, 1998). Temperature and duration requirements vary widely between species known to respond to treatment of this type. In our study, we exposed mericarps of two *Nolana* species (*N. humifusa* and *N. rupicola*) to cool temperatures of 12°C for two to 12 weeks. Limited time and availability of mericarps prevented execution of a more complete study of response to multiple temperature ranges and durations in *Nolana*.

Cold stratification was unsuccessful in inducing germination in our study. No seedlings germinated from a total of 320 mericarps exposed to cold temperature treatment. For the control with 40 mericarps per species sown without a cold treatment, only three seedlings of *N. humifusa* and none of *N. rupicola* germinated. With the low germination rate it is impossible to ascertain whether 12°C cold stratification may be detrimental to germination in these species. Temperatures of 12°C or lower are not unusual in winter months or at nights in the natural desert environment where *Nolana* can be found. It is unlikely that 12°C temperature reduced viability of the seed, but similarly to warm temperature (50°C), cold may enhance dormancy in these species.

Sterile culture of immature seed

Often, chemicals responsible for imposing endogenous dormancies are formed during late stages of seed maturation. In some species such as *Ophrys* and *Acer* it has been possible to avoid complications of seed dormancy by germinating immature seed prior to formation of dormancy-inducing chemicals (Kitsaki et al., 2004; Pinfield and Gwarazimba, 1992). This may be accomplished by sterile culture of intact immature seeds on a rich culture medium, or by embryo rescue where embryos are excised from immature seeds at various stages of development and germinated in sterile culture.

We explored the possibility that dormancy-inducing compounds are produced during late stages of fruit development in *Nolana* by comparing germination of seed from non-ripe *Nolana* fruits to that of seed from ripe fruits. Because we have been unsuccessful in excising intact *Nolana* seeds from mericarps, we cultured complete mericarps on a rich embryo rescue medium (Appendix C).

We found that seed from non-ripe fruits did not germinate under the experimental conditions employed. Moreover, the experimental conditions did not increase germination rates of seed from ripe fruits above those typically achieved under normal greenhouse conditions. No seedlings germinated from mericarps harvested prior to ripening or from mericarps harvested and sown at time of ripening. In total, six seedlings germinated (five *N. humifusa,* one *N. rupicola*), and all were obtained from mericarps harvested at ripe stage and stored for one week prior to sowing in culture. Based on these results, sterile culture of immature seed does not appear to be a viable means of germinating *Nolana* seed.

Conclusions

These studies of *Nolana* seed germination are not intended to be a comprehensive analysis of germination requirements of the studied species. The purpose of these studies is to identify treatments which exhibit potential in increasing germination rates in *Nolana*, with the ultimate goal of achieving reliably high germination rates which will increase efficiency and success of our *Nolana* research and breeding programs.

We were unable to identify a treatment which effectively increases *Nolana* germination rates to ideally high levels. However, we made several significant discoveries and made progress towards identifying an effective treatment. Analysis of

mericarp characteristics by scanning electron microscopy revealed presence of a tube of cells leading through funicular germination plugs toward enclosed seeds. Subsequent study revealed that the tube functions as a direct path of water uptake to individual seeds within the mericarp. It was found that the mericarp coating does not inhibit water uptake to the seeds. Imbibition occurred and germination plugs loosened without requiring scarification. This finding is significant as it narrows the field of focus for subsequent studies of factors effecting *Nolana* germination.

As the focus of our studies shifted towards non-physical germination barriers, evidence suggested that low germination rates were likely due to a physiological dormancy. Gibberellic acid was identified as a moderately successful treatment in inducing germination in some *Nolana* species, although germination rates remained far below ideal. Modification of soaking times to reflect individual species' rates of imbibition did not increase success of gibberellic acid treatment. Additionally, we found that germination rates tend to be higher in seed that has been stored for many months versus seed which has been recently harvested. Our attempts to break down dormancy in short periods by artificially ageing seed with exposure to warm temperatures showed variable success between species. Overall, species exhibit great variability in response to germination treatments. Several treatments were identified which show potential in some species, but showed no response in others. It is likely that innate differences between species will require development of individualized treatments for each species.

X-ray analysis proved to be a valuable tool in our studies of *Nolana* germination. We were able to use this technology to non-destructively view seeds within mericarps, allowing us to make seed counts for the first time in our studies. We found that most

mericarps contain at least one seed, and many contain several. This finding verifies that our expectations of achieving germination rates in excess of one seedling per mericarp are reasonable. Moreover, we have achieved the first documented report of average number of seeds per mericarp for eight *Nolana* species.

These studies have contributed greatly to our understanding of *Nolana* seed germination, but there is still much to be learned in this area. A suitable method of germinating *Nolana* seed has not been discovered, and germination rates remain well below ideal. However, we have ruled out that low germination rates are due to physical barriers, or in most cases, due to low seed set. We have narrowed the number of germination treatments yet to be explored and have identified a number of treatments which may be optimized.

Table 3.1 Species, collection/source information, and species habit of *Nolana* species used in germination studies, and germination summary for all experiments. All mericarps with the exceptions of *N. paradoxa* var. 'Blue Bird' and wild collected *N. paradoxa* (used in exp #1) were produced by manual hybridization at UNH between two or more accessions within each species.

			Overall germination rates for each experiment (seedlings germinated/mericarp sown)									
Species	Collection location of parent accessions / source	Species habit[z]	1	2[y]	3	4	5	6[y]	7[x]	8	9	10
N. adansonii	17°01' S, 72°02' W, 0-5 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo	erect herbaceous annual	-----	X	0.01		-----	X	0.10	0.10	-----	-----
N. aticoana	15°47' S, 74°21' W, 450-480 masl, Peru, Arequipa, Lomas de Atiquipa	herbaceous perennial	0.20	X	0.14		0.09	X	0.06	0.17	-----	-----
N. elegans	25°26' S, 70°26' W, 890 masl, Chile, Region II, Prov. Antofagasta, Cerro Perales, near Taltal	procumbent herbaceous annual	0	X	0.03		-----	X	0.02	0.06	-----	-----
N. humifusa	12°11' S, 76°48' W, 170 masl, Peru, Lima, Lomas de Pachacamac	herbaceous annual	0.25	X	0.04		-----	X	0.03	0.08	0.02	0.04
N. ivaniana	17°01' S, 72°02' W, 5-10 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo	erect herbaceous annual	0	X	0		-----	X	0.02	0.11	-----	-----
N. laxa	11°58' S, 76°46' W, 670-700 masl, Peru, Lima, Los Condores	erect herbaceous annual	0	X	0.00		-----	X	-----	0.01	-----	-----
N. paradoxa	30°06'S - 42°40' W, Chile, central to southern regions	herbaceous annual	0.29	X	-----	See Table 3.4 for details	-----	--	-----	-----	-----	-----
N. plicata	15°47' S, 74°21' W, 400 masl, Peru, Arequipa, Lomas de Atiquipa	herbaceous perennial	0.08	X	0.09		-----	X	0.01	0.05	-----	0
N. rupicola	26°01' S, 70°36' W, 720-780 masl, Chile, Region III, Atacama, Prov. Chanaral, Parque Nacional Pan de Azucar. Las Lomitas	herbaceous perennial	0	X	0.01		0	X	0.02	0.03	0	0.00
N. paradoxa var. 'Blue Bird'	Commercial seed purchased from J.L.Hudson, Seedsman, La Honda, California, USA, 2004	herbaceous annual	0.49	---	-----		-----	--	-----	-----	-----	-----

[z]Adapted from Tago-Nakazawa and Dillon, 1999

[y]Germination rates were not evaluated in Experiments 2 and 6. X indicates species used in these experiments.

[x]Germination rate for this experiment calculated based on number of seeds sown rather than mericarps sown.

Table 3.2 Chi-square goodness of fit analyses of germination in Experiment 3 - Survey of chemical, hormonal, and environmental treatments. Expected values assume no treatment effects and represent equal distribution of the total number of germinated seedlings across all treatments.

Species			Media-treatments A-1	A-2	A-3	A-4	A-5	Total
all species	p=0.000	Observed	13	10	40	1	11	75
		Expected	15	15	15	15	15	75
		$(O-E)^2/E$	0.3	1.7	41.7	13.1	1.1	57.7
N. adansonii	p=0.736	Observed	0	0	1	1	1	3
		Expected	0.6	0.6	0.6	0.6	0.6	3
		$(O-E)^2/E$	0.6	0.6	0.3	0.3	0.3	2.0
N. aricoana	p=0.044	Observed	10	9	6	0	6	31
		Expected	6.2	6.2	6.2	6.2	6.2	31
		$(O-E)^2/E$	2.3	1.3	0.0	6.2	0.0	9.8
N. elegans	p=0.004	Observed	1	0	5	0	0	6
		Expected	1.2	1.2	1.2	1.2	1.2	6
		$(O-E)^2/E$	0.0	1.2	12.0	1.2	1.2	15.7
N. humifusa	p=0.000	Observed	0	0	12	0	0	12
		Expected	2.4	2.4	2.4	2.4	2.4	12
		$(O-E)^2/E$	2.4	2.4	38.4	2.4	2.4	48.0
N. ivaniana	NA	Observed	0	0	0	0	0	0
		Expected	0	0	0	0	0	0
		$(O-E)^2/E$	NA	NA	NA	NA	NA	
N. laxa	NA	Observed	0	0	0	0	0	0
		Expected	0	0	0	0	0	0
		$(O-E)^2/E$	NA	NA	NA	NA	NA	
N. plicata	p=0.000	Observed	2	1	14	0	4	21
		Expected	4.2	4.2	4.2	4.2	4.2	21
		$(O-E)^2/E$	1.2	2.4	22.9	4.2	0.0	30.7
N. rupicola	p=0.092	Observed	0	0	2	0	0	2
		Expected	0.4	0.4	0.4	0.4	0.4	2
		$(O-E)^2/E$	0.4	0.4	6.4	0.4	0.4	8.0

Species			Liquid-treatments B-1	B-2	B-3	B-4	Total
all species	p=0.029	Observed	1	9	3	3	16
		Expected	4	4	4	4	16
		$(O-E)^2/E$	2.3	6.3	0.3	0.3	9.0
N. adansonii	p=0.391	Observed	0	0	0	1	1
		Expected	0.3	0.3	0.3	0.3	1
		$(O-E)^2/E$	0.3	0.3	0.3	2.3	3.0
N. aricoana	p=0.000	Observed	0	7	0	0	7
		Expected	1.8	1.8	1.8	1.8	7
		$(O-E)^2/E$	1.8	15.8	1.8	1.8	21.0
N. elegans	p=0.801	Observed	0	1	1	1	3
		Expected	0.8	0.8	0.8	0.8	3
		$(O-E)^2/E$	0.8	0.1	0.1	0.1	1.0
N. humifusa	NA	Observed	0	0	0	0	0
		Expected	0	0	0	0	0
		$(O-E)^2/E$	NA	NA	NA	NA	
N. ivaniana	NA	Observed	0	0	0	0	0
		Expected	0	0	0	0	0
		$(O-E)^2/E$	NA	NA	NA	NA	
N. laxa	p=0.391	Observed	0	0	1	0	1
		Expected	0.3	0.3	0.3	0.3	1
		$(O-E)^2/E$	0.25	0.25	2.25	0.25	3.0
N. plicata	p=1.000	Observed	1	1	1	1	4
		Expected	1.0	1.0	1.0	1.0	4
		$(O-E)^2/E$	0.0	0.0	0.0	0.0	0.0
N. rupicola	NA	Observed	0	0	0	0	0
		Expected	0	0	0	0	0
		$(O-E)^2/E$	NA	NA	NA	NA	

Table 3.3 Germination of *Nolana* mericarps exposed to pre-sowing soaking treatments. *N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata,* and *N. rupicola* were exposed to treatments 1, 4, and 5 only due to limited mericarp supply. Number of seedlings per treatment germinated at 38 weeks post sowing is reported. Subscripts indicate seedlings germinated during treatment, prior to sowing.

	N. paradoxa 'Blue Bird'	*N. paradoxa*	*N. aticoana*	*N. elegans*	*N. humifusa*	*N. ivaniana*	*N. laxa*	*N. plicata*	*N. rupicola*
	Mericarps sown per treatment								
Treatment	20	20	11	17	20	12	13	19	20
1. No treatment	10	3	7	0	6	0	0	4	0
2. 24 hour soak	14	4							
3. 24 hour soak x2	14_1	7							
4. 5 day soak	14_8	8	4_1	0	2	0	0	1	0
5. 5 day soak, 4 day soak	10_9	11	1	0	7	0	0	0	0
6. Sulfuric acid	7	8							
7. Boiling / ice water	0	0							
Germination rate[z]	0.49	0.29	0.20	0	0.25	0	0	0.08	0

[z]Germination rate calculated as seedlings germinated per mericarp sown.

Table 3.4 Germination of eight species of *Nolana* in response to nine chemical or environmental germination treatments. Values represent number of seedlings germinating from 30 mericarps per treatment per species.

		Treatment	Species								Total seedlings germinated	Treatment germination rate[x]
			N. adansonii	*N. aticoana*	*N. elegans*	*N. humifusa*	*N. ivaniana*	*N. laxa*	*N. plicata*	*N. rupicola*		
Media-based	A-1	no treatment	0	10	1	0	0	0	2	0	13	0.05
	A-2	nitric acid	0	9	0	0	0	0	1	0	10	0.04
	A-3	gibberellic acid	1	6	5[z]	12[z]	0	0	14[z]	2[z]	40[z]	0.17
	A-4	sulfuric acid	1	0	0	0	0	0	0	0	1	0.00
	A-5	temperature	1	6	0	0	0	0	4	0	11	0.05
Liquid-based	B-1	distilled water	0	0	0	0	0	0	1[y]	0	1	0.00
	B-2	10mM ethephon	0	7	1	0	0	0	1	0	9	0.04
	B-3	1*u*M ethephon	0	0	1	0	0	1[y]	1	0	3	0.01
	B-4	potassium nitrate	1[y]	0	1	0	0	0	1	0	3	0.01
		Species germination rate[w].	0.01	0.14	0.03	0.04	0	0.00	0.09	0.01		

[z]Germination of gibberellic acid treated mericarps is notably higher than that of all other treatments.

[y]Seedling germinated after sowing, rather than during soaking.

[x]Treatment germination rate calculated as the average number of seedlings germinated per mericarp from all species combined (240 mericarps sown per treatment).

[w]Species germination rate calculated as the average number of seedlings germinated per mericarp from all treatments combined (270 mericarps sown per species).

Table 3.5 Germination of *Nolana* seed following three storage durations (6, 20, or 24 months) or without storage (0 months). In each case, germination from stored mericarps is compared to that of freshly produced replicates of identical parentage.

Species or cross		Mericarps sown per age group	Seedlings germinated	
			0 months	6 months
N. adansonii		45	5	2
N. aticoana		45	0	4
N. elegans		30	0	1
N. humifusa		30	0	0
N. ivaniana		15	1	0
N. laxa		15	0	0
N. plicata		15	0	3
N. rupicola		90	0	0
	Total:	285	6	10
	Germination rate[z]:		0.02	0.04
			0 months	20 months
N. humifusa		30	0	0
N. ivaniana		15	0	0
N. laxa		15	0	0
N. rupicola		90	0	0
	Total:	150	0	0
	Germination rate:		0	0
			0 months	24 months
N. aticoana x N. plicata		60	0	60
N. humifusa x N. aticoana		60	0	24
N. humifusa x N. plicata		60	1	23
N. plicata x N. aticoana		15	0	0
N. plicata x N. humifusa		15	0	9
	Total:	210	1	116
	Germination rate:		0.00	0.55

[a]Germination rate = average seedlings per mericarp.

Table 3.6 Rates of imbibition in eight *Nolana* species.
Number of mericarps out of five mericarps observed per day per species exhibiting imbibition of dye solution into at least one enclosed seed on each of 14 days of soaking. Boxes indicate the first day on which at least four out of five mericarps of a species exhibited imbibition. Imbibition indices are calculated as the average number of imbibed mericarps per day and illustrate relative rates of imbibition between species.

Species	Imbibed mericarps: Days of soaking 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Imbibition index	Species-specific soaking durations[z]
N. adansonii	1	0	1	2	3	2	2	**4**	3	5	4	4	3	5	2.79	10
N. rupicola	1	1	2	**4**	4	3	4	4	4	3	4	5	5	5	3.50	6
N. elegans	2	**4**	4	4	3	4	4	5	4	4	4	5	4	5	4.00	4
N. ivaniana	0	3	3	**5**	4	5	5	4	5	4	5	5	5	5	4.14	6
N. laxa	3	**4**	3	5	4	5	5	5	5	4	5	5	5	5	4.50	4
N. aticoana	**4**	5	4	5	5	4	5	5	4	5	5	5	5	5	4.71	3
N. humifusa	**5**	5	5	5	5	4	5	4	5	5	5	5	5	5	4.86	3
N. plicata	**5**	5	5	5	4	5	5	5	5	5	5	5	5	5	4.93	3

[z]Species-specific soaking durations indicate target soaking times to ensure imbibition. Calculated as two days plus the number of days of soaking required for at least four out of five mericarps to exhibit imbibition in imbibition rate study.

Table 3.7 Counts of seed in mericarps of *Nolana* developed through artificial hybridization. Seed counts are estimated from x-ray analysis of mericarps.

		Number of mericarps analyzed	Average full seeds per mericarp[z]	Average seed cavities per mericarp (full, empty, and abnormal)[y]	Percent cavities with supposed full seeds	Percent mericarps with one or more supposed full seeds
Species	*N. adansonii*	139	0.86 ± 0.39	1.06 ± 0.23	81%	84%
	N. aticoana	228	1.88 ± 1.81	3.94 ± 1.24	48%	64%
	N. elegans	112	2.42 ± 1.80	3.97 ± 2.22	61%	94%
	N. humifusa	224	2.54 ± 1.40	4.27 ± 0.94	59%	93%
	N. ivaniana	50	4.20 ± 1.80	5.16 ± 1.49	81%	94%
	N. plicata	89	5.03 ± 2.97	7.43 ± 3.03	68%	97%
	N. rupicola	82	1.12 ± 1.00	1.62 ± 1.04	69%	77%
Hybrid families	*N. adansonii* x *N. aticoana*	93	0.17 ± 0.46	1.05 ± 0.23	16%	14%
	N. adansonii x *N. plicata*	177	0.24 ± 0.46	1.07 ± 0.26	22%	22%
	N. aticoana x *N. adansonii*	38	1.68 ± 1.04	4.08 ± 0.85	41%	87%
	N. aticoana x *N. humifusa*	27	2.22 ± 1.45	3.78 ± 1.31	59%	89%
	N. aticoana x *N. laxa*	100	2.48 ± 1.24	4.06 ± 1.56	61%	96%
	N. aticoana x *N. plicata*	84	3.44 ± 1.80	4.31 ± 1.78	80%	96%
	N. elegans x *N. aticoana*	82	0.46 ± 0.61	2.09 ± 1.11	22%	40%
	N. elegans x *N. ivaniana*	51	0.27 ± 0.45	1.49 ± 0.73	18%	27%
	N. elegans x *N. laxa*	27	0.07 ± 0.27	1.63 ± 0.74	5%	7%
	N. elegans x *N. plicata*	35	0.63 ± 0.65	2.26 ± 1.27	28%	57%
	N. elegans x *N. rupicola*	286	1.82 ± 1.52	2.77 ± 1.97	66%	86%
	N. humifusa x *N. adansonii*	63	0.95 ± 0.94	2.86 ± 1.00	33%	67%
	N. humifusa x *N. aticoana*	109	2.18 ± 1.55	3.69 ± 0.99	59%	83%
	N. humifusa x *N. elegans*	14	1.00 ± 1.78	3.07 ± 1.44	33%	71%
	N. humifusa x *N. ivaniana*	182	2.01 ± 1.51	3.75 ± 1.15	54%	83%
	N. humifusa x *N. laxa*	180	2.58 ± 1.46	3.69 ± 0.95	70%	89%
	N. humifusa x *N. plicata*	120	2.58 ± 1.73	3.98 ± 1.58	65%	90%
	N. humifusa x *N. rupicola*	60	1.12 ± 1.44	3.25 ± 1.05	34%	60%
	N. ivaniana x *N. aticoana*	39	2.15 ± 1.55	2.15 ± 1.86	100%	87%
	N. ivaniana x *N. elegans*	19	3.68 ± 1.77	4.74 ± 1.94	78%	95%
	N. ivaniana x *N. laxa*	214	3.05 ± 2.06	4.14 ± 2.04	74%	92%
	N. ivaniana x *N. plicata*	88	2.77 ± 2.20	4.08 ± 1.67	68%	76%
	N. ivaniana x *N. rupicola*	78	4.00 ± 1.74	4.56 ± 1.70	88%	100%
	N. plicata x *N. aticoana*	115	3.57 ± 2.15	4.97 ± 2.57	72%	97%
	N. plicata x *N. humifusa*	24	2.08 ± 1.41	4.50 ± 0.72	46%	92%
	N. plicata x *N. laxa*	12	0	8.58 ± 3.23	0	0
	N. rupicola x *N. elegans*	277	1.48 ± 1.08	1.94 ± 1.23	76%	85%

[z]Designation of 'full seed' based on contrast in x-ray image. This value represents the number of cavities visible in the x-ray image appearing full. This may not be an accurate representation of viable seeds present. Value represents the average count of all analyzed mericarps +/- standard deviation. Value may represent multiple families within the indicated species pair.

[y]Seed cavities were identified in the x-ray images based on contrast. This count includes those cavities appearing to contain full seeds, those which appear empty, and those which appear to contain abnormal seeds. Value represents the average count of all analyzed mericarps +/- standard deviation. Value may represent multiple families within the indicated species pair.

Table 3.8 Germination of *Nolana* seed following x-ray analysis. Germination rates represent number of seedlings germinated from the estimated number of seeds sown as determined by x-ray analysis of mericarps.

Species or hybrid family[z]	Seeds per mericarp		Mericarps sown	Estimated seeds sown	Seedlings germinated	Estimated germination rate[w]
	Mean[y]	Range[x]				
N. adansonii	1.0	1-2	100	100	10	0.10
N. aticoana	3.1	1-7	140	434	24	0.06
N. elegans	2.7	1-7	100	270	6	0.02
N. humifusa	2.7	1-5	200	540	17	0.03
N. ivaniana	4.5	1-8	46	207	5	0.02
N. plicata	5.3	1-4	80	424	4	0.01
N. rupicola	1.5	1-13	62	93	2	0.02
N. adansonii x *N. aticoana*	0.2	0-2	106	18	0	0
N. adansonii x *N. plicata*	0.2	0-2	176	42	0	0
N. aticoana x *N. adansonii*	1.7	0-4	60	101	19	0.19
N. aticoana x *N. humifusa*	2.2	0-6	25	56	4	0.07
N. aticoana x *N. laxa*	2.5	0-5	93	231	0	0
N. aticoana x *N. plicata*	3.4	0-10	111	382	52	0.14
N. elegans x *N. aticoana*	0.5	0-2	109	50	34	0.68
N. elegans x *N. ivaniana*	0.3	0-1	53	14	9	0.63
N. elegans x *N. laxa*	0.1	0-1	32	2	1	0.45
N. elegans x *N. plicata*	0.6	0-3	40	25	15	0.60
N. elegans x *N. rupicola*	1.8	0-8	466	848	101	0.12
N. humifusa x *N. adansonii*	1.0	0-5	64	61	4	0.07
N. humifusa x *N. aticoana*	2.2	0-6	98	214	17	0.08
N. humifusa x *N. elegans*	1.0	0-2	15	15	0	0
N. humifusa x *N. ivaniana*	2.0	0-9	220	442	3	0.01
N. humifusa x *N. laxa*	2.6	0-5	221	570	1	0.00
N. humifusa x *N. plicata*	2.6	0-8	110	284	54	0.19
N. humifusa x *N. rupicola*	1.1	0-9	76	85	0	0
N. ivaniana x *N. aticoana*	2.2	0-5	42	90	8	0.09
N. ivaniana x *N. elegans*	3.7	0-6	20	74	0	0
N. ivaniana x *N. laxa*	3.1	0-8	204	622	4	0.01
N. ivaniana x *N. plicata*	2.8	0-7	104	288	28	0.10
N. ivaniana x *N. rupicola*	4.0	1-7	94	376	18	0.05
N. plicata x *N. aticoana*	3.6	0-11	179	639	8	0.01
N. plicata x *N. humifusa*	2.1	0-5	25	52	0	0
N. plicata x *N. laxa*	0.0	0	13	0	0	0
N. rupicola x *N. elegans*	1.5	0-7	256	379	0	0
Total	1.8	0-13	3740	8028	448	0.11

[z]Mericarps produced through artificial interspecific hybridization at the University of New Hampshire 2005-2006. Additional details of hybrid parentage are presented in Appendix B.

[y]Seeds per mericarp estimates made by x-ray analysis of a random set of mericarps from each species or hybrid family. Details are presented in Table 3.6.

[x]Mericarps of *Nolana* species containing zero seeds were present in x-rayed samples, but were not sown and are therefore not represented in this table.

[w]Germination rate calculated as the number of seedlings germinated divided by the estimated number of seeds sown.

Table 3.9 Instances of germination of multiple seedlings from single *Nolana* mericarps.

Species or hybrid family[z]	Mericarps sown	Average seeds per mericarp[y]	Mericarps with 1 seedling germinated	Mericarps with 2 seedlings germinated	Mericarps with 3 seedlings germinated	Days between germination events within mericarps[x]
N. adansonii	100	1.02	8	1		30
N. aticoana	140	3.03	15	3	1	0, 1, 1, 3, 9
N. humifusa	200	2.72	9	4		12, 14, 32, 79
N. aticoana x N. adansonii	54	1.68	17	1		2
N. aticoana x *N. plicata*	115	3.44	40	6		0, 0, 0, 1, 2, 3
N. elegans x *N. aticoana*	55	0.46	32	1		6
N. elegans x *N. rupicola*	459	1.82	88	9		0, 0, 1, 1, 1, 1, 1, 2, 2
N. humifusa x *N. aticoana*	98	2.18	16	1		0
N. humifusa x *N. ivaniana*	172	2.01	1	1		0
N. humifusa x *N. plicata*	93	2.58	29	11	1	0, 0, 0, 0, 0, 0, 1, 1, 1, 2, 2, 2, 7
N. ivaniana x *N. laxa*	173	3.05	2	1		2
N. ivaniana x *N. plicata*	94	2.77	17	4	2	0, 0, 0, 2, 3, 4, 8, 14
N. ivaniana x *N. rupicola*	93	4	9	3	1	2, 2, 4, 10, 32

[z]Only those species or hybrids with instances of multiple seedlings germinating from single mericarps are reported.

[y]Seeds per mericarp estimates made by x-ray analysis of a random set of mericarps from each cross.

[x]Number of days between germination events in cases where multiple seedlings germinated from a single mericarp.

Table 3.10 Germination of *Nolana* in response to combinations and modifications of gbberellic acid and warm temperature treatments.

Treatment	*N. adansonii*	*N. aticoana*	*N. elegans*	*N. humifusa*	*N. ivaniana*	*N. laxa*	*N. plicata*	*N. rupicola*	Overall germination rate (seedlings per mericarp)
Control – no treatment	1	4	2	2	0[x]	0[z]	0[z]	1[x]	0.08
Gibberellic acid soak for species-specific duration (3-10 days)	3	5	3	2	3[x]	0[z]	1[z]	0[y]	0.13
Warm treatment (35°C) for 7 weeks while sown in moist media	3	0	0	4	2[x]	0[z]	2[z]	0[y]	0.08
Warm treatment (35°C) for 7 weeks at 75% relative humidity	0	6	0	0	0[x]	0[z]	0	0[z]	0.04
GA_3 (species-specific) followed by 7 weeks at 35°C	3	1	1	1	0[x]	1[z]	1[z]	1[z]	0.07
35°C at 75% RH for 7 weeks followed by GA_3 (species-specific)		4		6					
GA_3 (species-specific) followed by 4 days at 50°C (moist)		4		1					
50°C for 2 days (dry)				0					
50°C for 4 days (dry)				0					
50°C for 6 days (dry)				0					
overall germination rate (seedlings per mericarp)	0.10	0.17	0.06	0.08	0.11	0.01	0.05	0.03	0.09

[z]15 mericarps treated
[y]12 mericarps treated
[x]9 mericarps treated

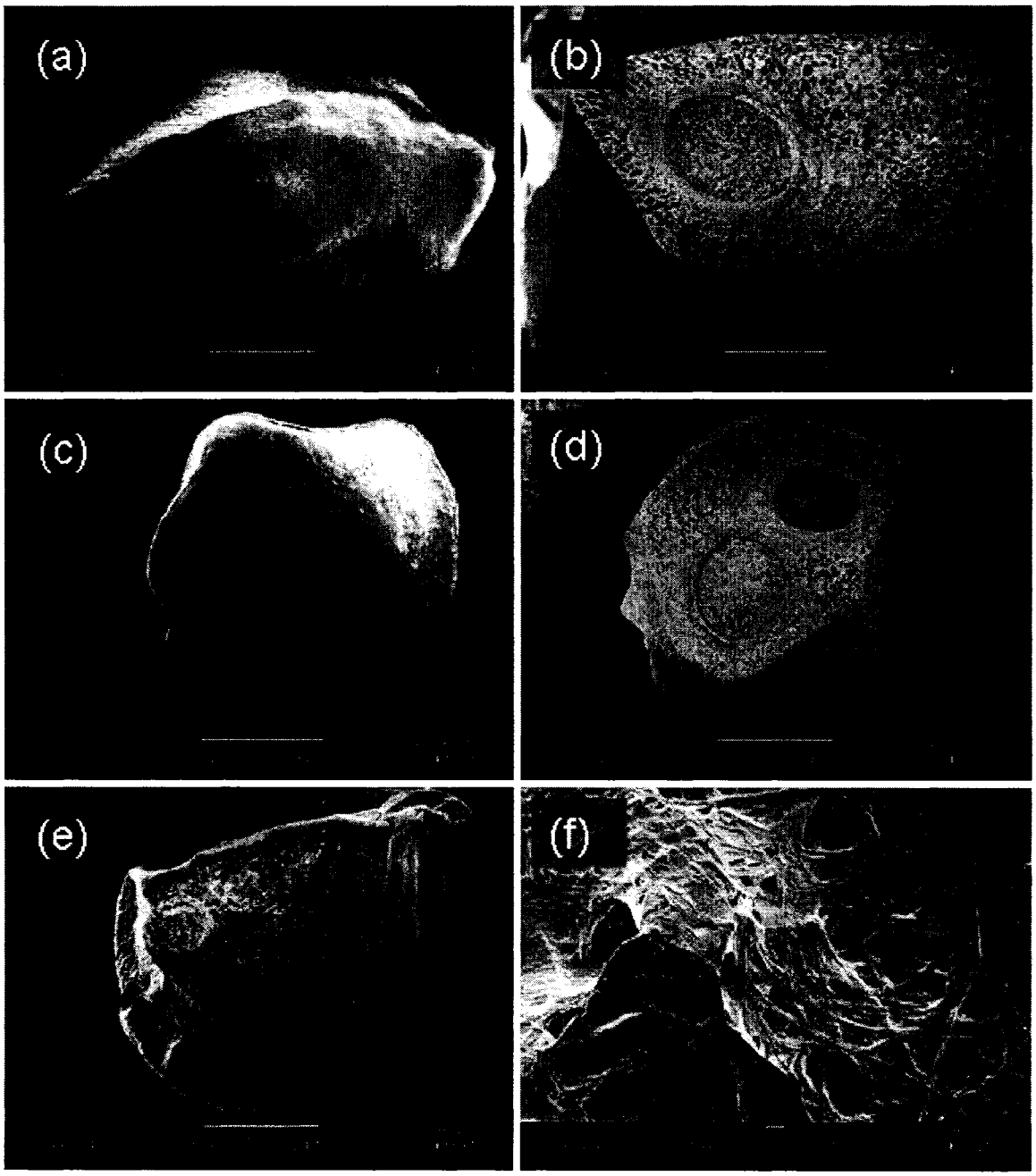


Figure 3.1 Scanning electron micrographs of *Nolana* mericarps. (a) *N. paradoxa*, no treatment. Surface coating is intact. (b) *N. paradoxa*, 30 min soak in concentrated sulfuric acid. Surface coating has been degraded exposing porous mericarp body tissue. (c) *N. paradoxa* var. 'Blue Bird', no treatment. Surface coating is intact. (d) *N. paradoxa* var. 'Blue Bird', 30 min soak in concentrated sulfuric acid. Surface coating has been degraded exposing porous mericarp body. One funicular germination plug is malformed. This deformity was present prior to treatment. (e) *N. humifusa*, no treatment. Surface coating is sparse. (f) Magnified view of surface coating over germination plug in *N. paradoxa*.

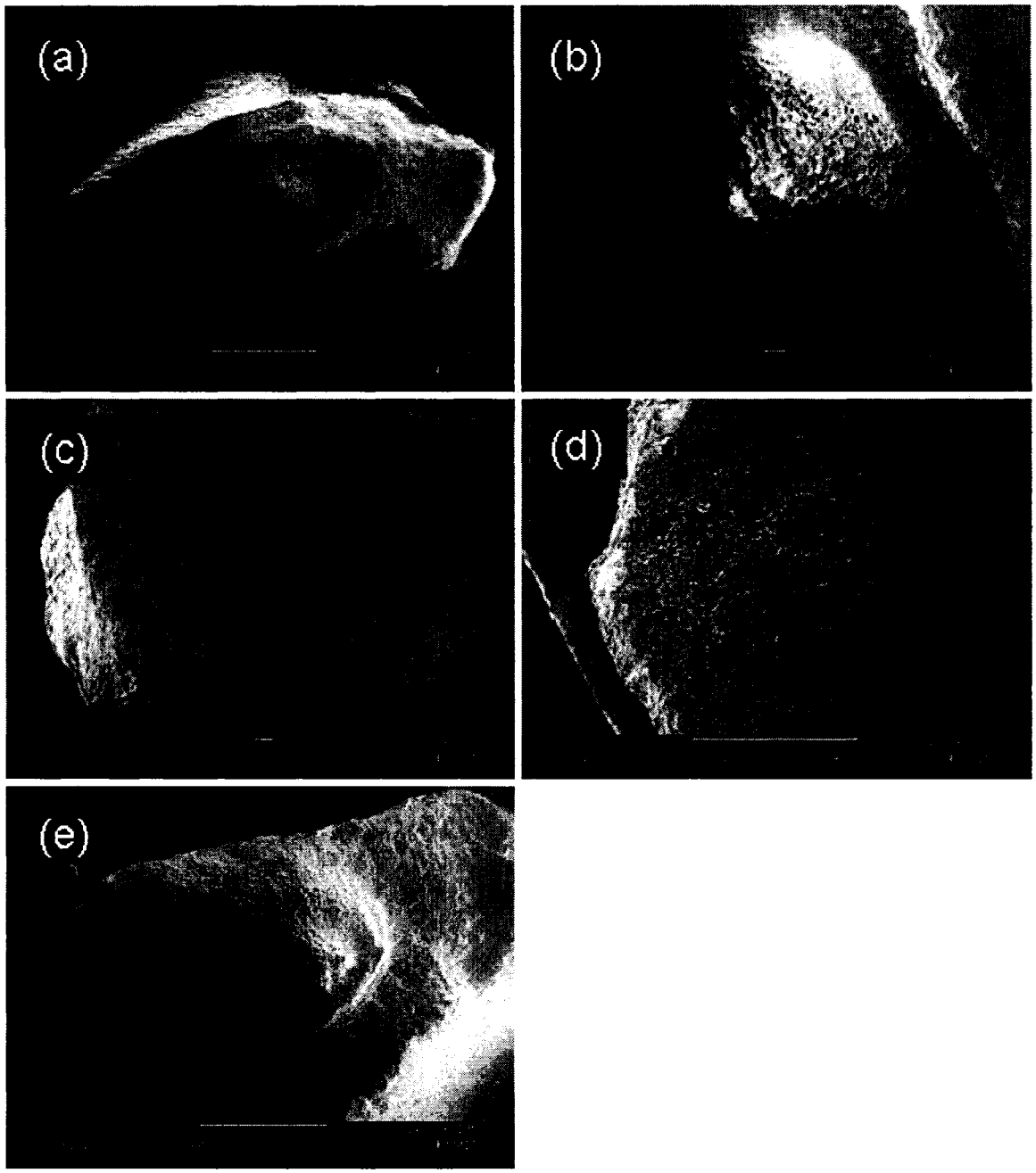


Figure 3.2 Scanning electron micrographs of *Nolana paradoxa* mericarps after soaking treatments. Integrity of surface coating is variable but does not appear to be a function of soaking treatments. Similar results were observed in other species. (a) No treatment. Surface coating is complete. (b) 24 h water soak. Surface coating is sparse, especially on the funicular germination plug. (c) 24 h water soak, 24 h dry, 24 h water soak. Surface coating is complete. (d) five day water soak. Surface coating is sparse, especially on the funicular germination plug. (e) five day water soak, 24 h dry, four day water soak. Surface coating is complete.

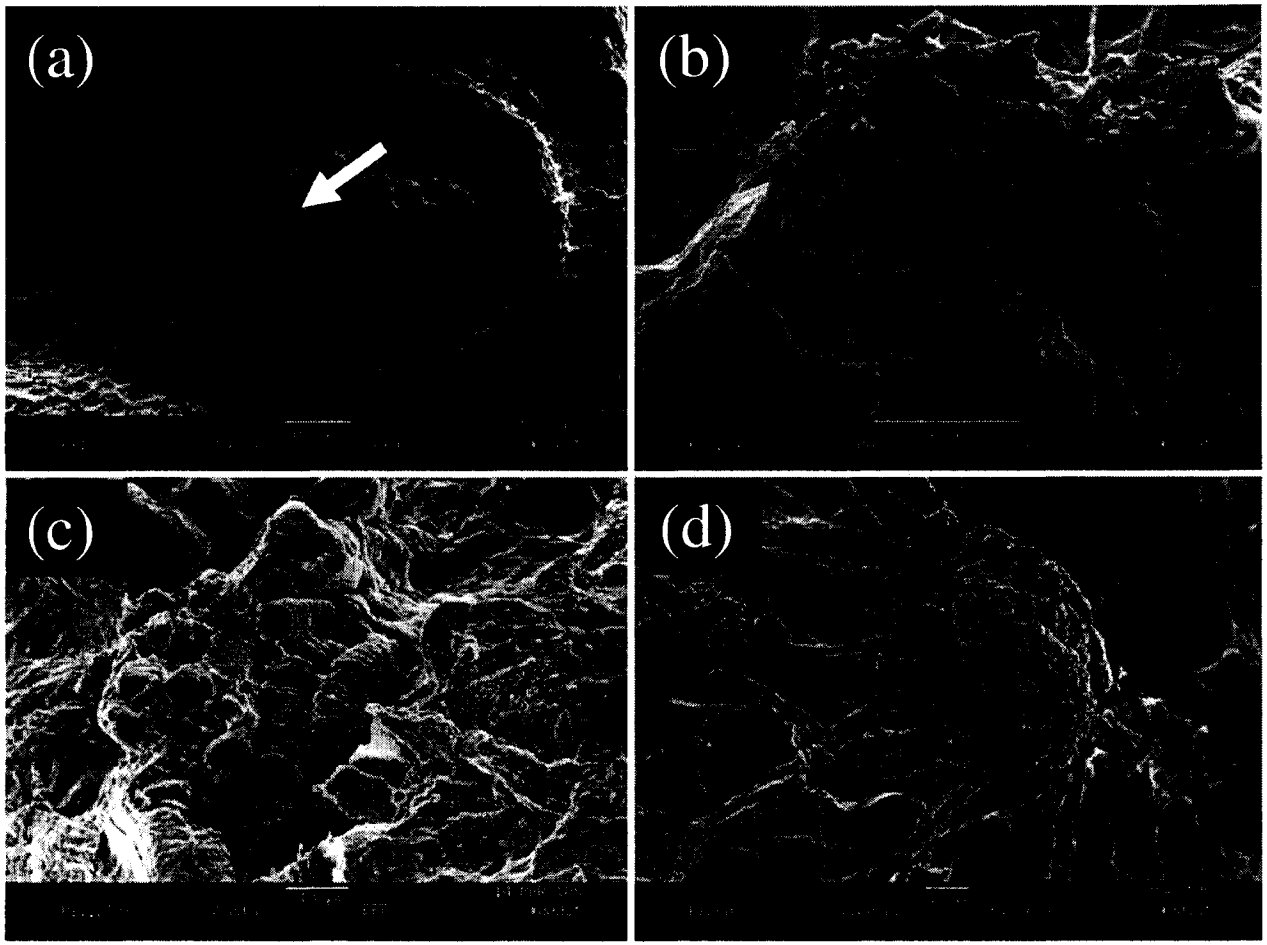


Figure 3.3 Scanning electron micrographs of funicles of *Nolana* mericarps. Funicles are found in the center of funicular germination plugs and are the path of water uptake to the enclosed seeds. (a) Funicular germination plug of *N. laxa*. Arrow points to funicle. (b) Magnification of funicle of *N. laxa*. (c) Funicle of *N. paradoxa* var. 'Blue Bird.' (d) Center of funicular germination plug of *N. plicata*. Few coiled tracheid cells of the funicle are visible, while others are presumably hidden beneath the surface coating.

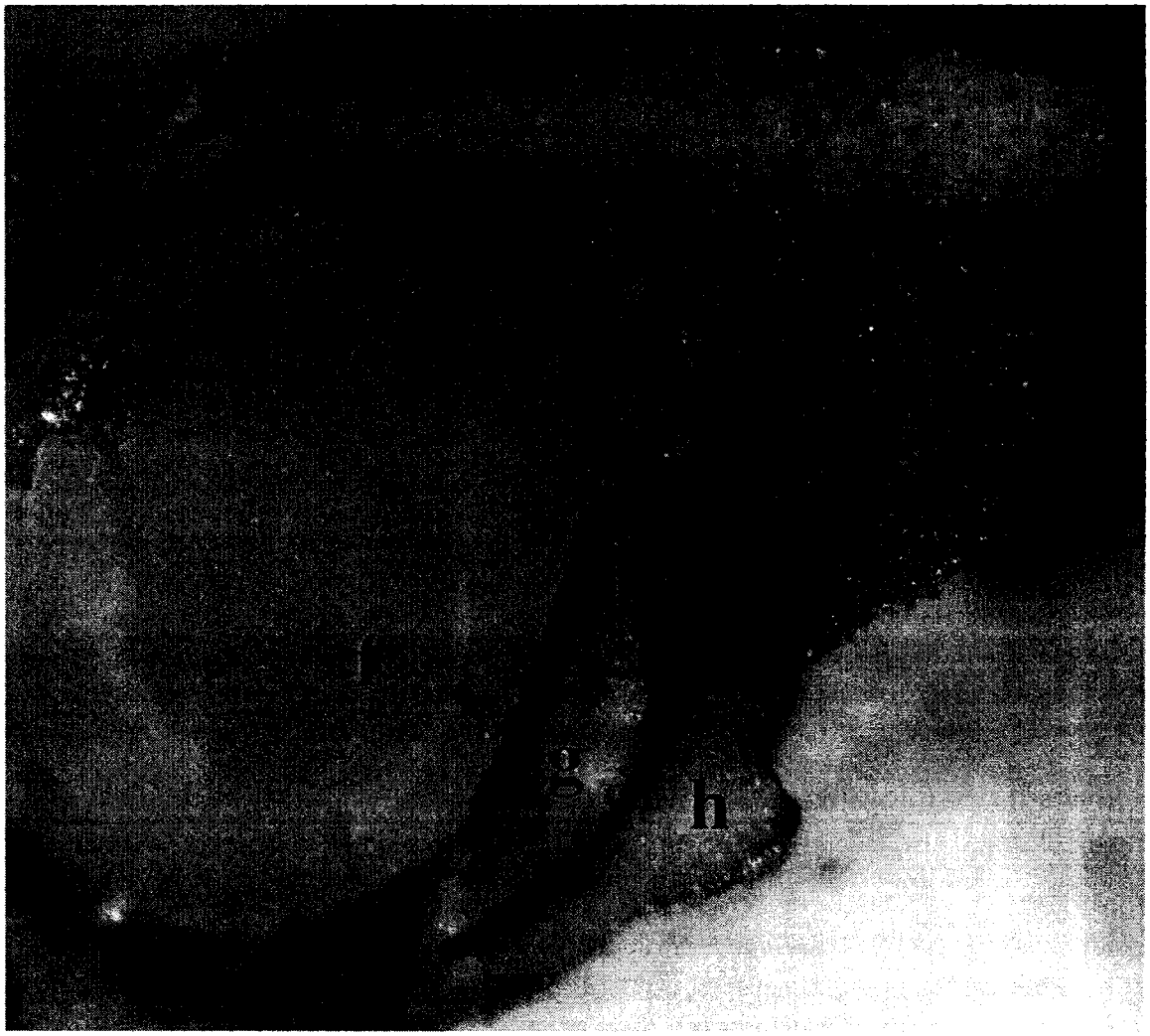


Figure 3.4 Cross-section of a mericarp of *N. paradoxa* which has been soaked in blue dye to identify the path of imbibition. Arrows indicate the path of dye movement over time as observed in a series of dissected mericarps. Dye entered the mericarp through the funicle (a), hydrated the lower layers of cells in the germination plug (b), spread along the edges of the germination plug (c) and across the seed coat (d). Other features indicated include the radicle end of the seed (e), the cotyledon end of the seed (f), the seed coat (g), mericarp outer coat (h), and the funicular germination plug (i).

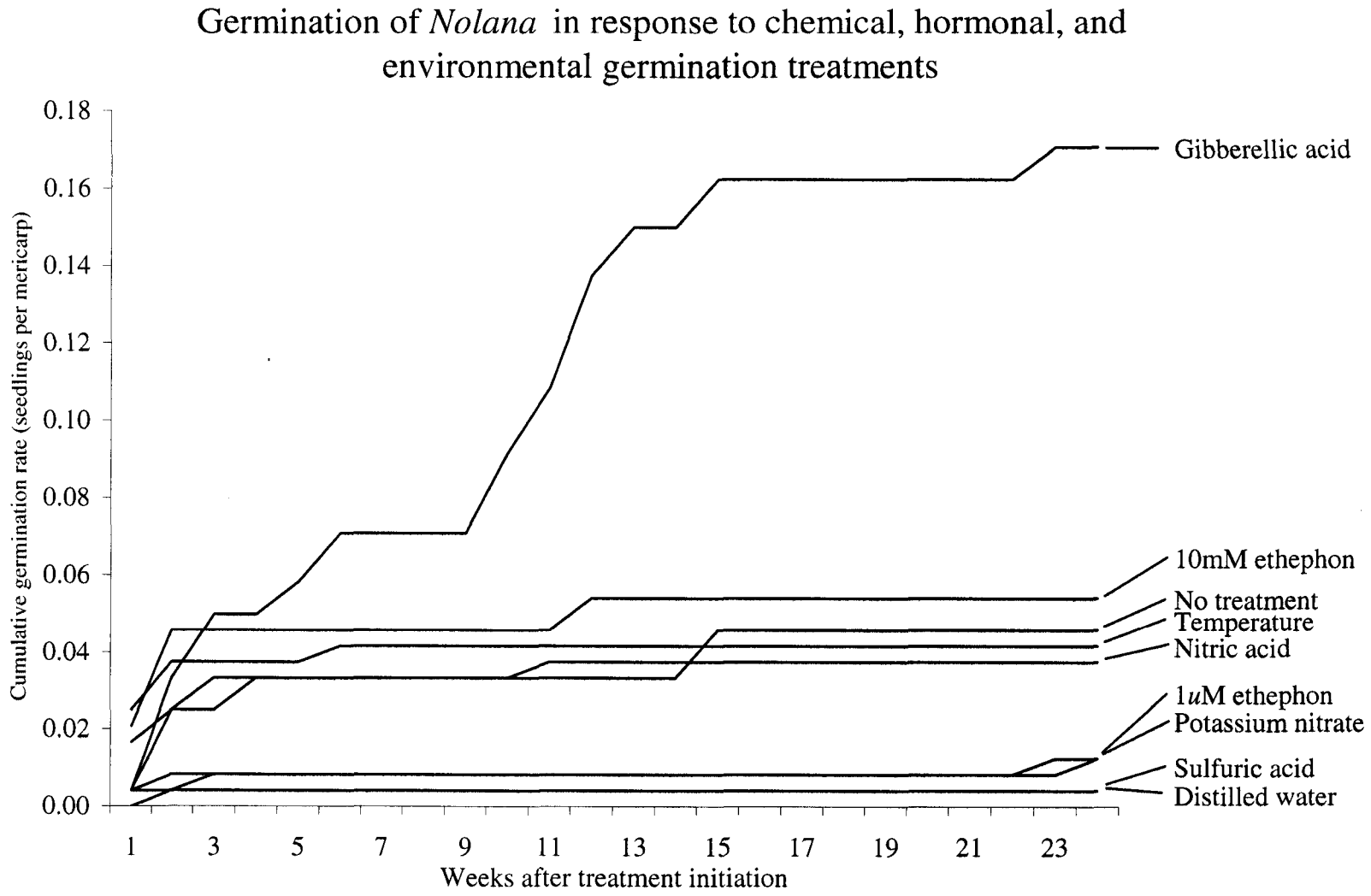


Figure 3.5 Germination of *Nolana* over time in response to nine germination treatments. Germination from mericarps treated with gibberellic acid continued for many weeks after germination in other treatments slowed or stopped.

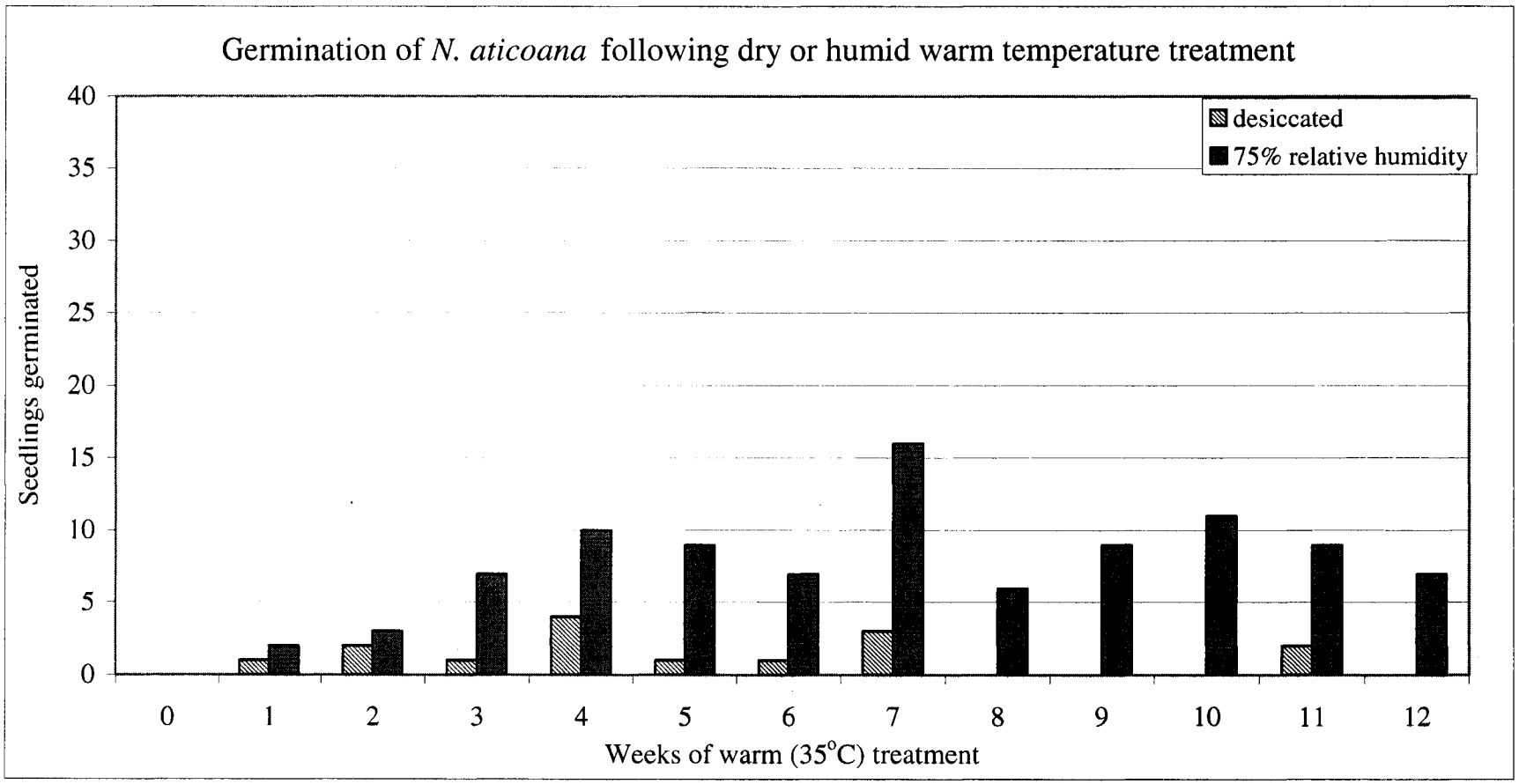


Figure 3.6 Germination of *N. aticoana* following warm temperature (35°C) storage for zero to 12 weeks in either a desiccated or humid environment. Forty mericarps were sown per treatment. Exposure of mericarps of *N. rupicola* to the same conditions resulted in no germination.

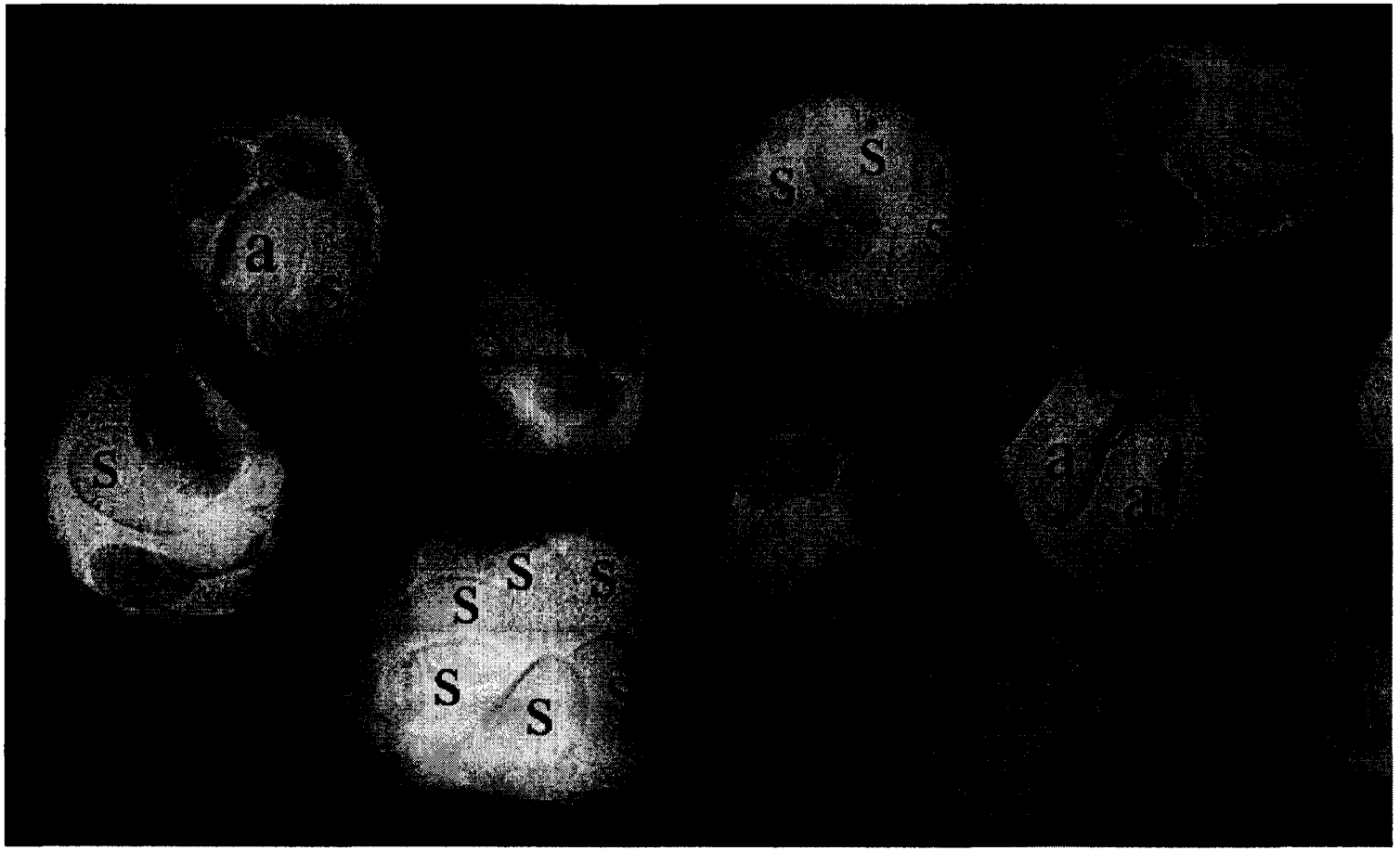


Figure 3.7 X-ray image of mericarps resulting from interspecific hybridization of *N. aticoana* x *N. adansonii*. Estimations of seed counts are made based on areas of differing contrast in the image. Cavities are labeled with 's' to designate a full seed, 'e' to designate an empty cavity, and 'a' to designate a seed which does not fill its cavity and therefore appears to be abnormally formed.

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APPENDICES

APPENDIX A. Parentage of mericarps used in natural aging experiment.

Mericarps from each storage treatment (24 months, 20 months, or six months storage) were replicated with fresh mericarps of identical genetic parentage. All mericarps were produced at UNH by artificial hybridization. Fifteen stored and 15 fresh mericarps were sown per hybridization.

	Species	Parentage[z]	Germ.[y]
Six month storage vs. zero month storage	*N. adansonii*	Ad2-2 x Ad4-1	5_f, 1_s
		Ad4-11 x Ad2-2	1_s
		Ad4-11 x Ad4-1	0
	N. aticoana	A2 x A13	2_s
		A3 x A2	2_s
		A13 x A3	0
	N. elegans	051-3 x 051-5	1_s
		Ele2 x Ele3	0
	N. humifusa	H28 x Hu1-2	0
		H28 x Hu9-4	0
	N. ivaniana	Iv2-1 x Iv2-2	1_f
	N. laxa	La1-2 x La1-4	0
	N. plicata	P5 x P11	3_s
	N. rupicola	Rup1 x Rup2	0
		Rup1 x Rup3	0
		Rup2 x Rup1	0
		Rup2 x Rup3	0
		Rup3 x Rup1	0
		Rup3 x Rup2	0
Twenty month storage vs. zero month storage	*N. humifusa*	Hu1-2 x Hu9-4	0
		Hu9-4 x H28	0
	N. ivaniana	Iv2-2 x Iv2-3	0
	N. laxa	La1-2 x La3-2	0
	N. rupicola	Rup1 x Rup2	0
		Rup1 x Rup3	0
		Rup2 x Rup1	0
		Rup2 x Rup3	0
		Rup3 x Rup1	0
		Rup3 x Rup2	0

	Cross	Parentage[z]	Germ.[y]
Twenty-four month storage vs. zero month storage	*N. aticoana x N. plicata*	A2 x P11	16_s
		A3 x P7	17_s
		A13 x P5	15_s
		A13 x P7	12_s
	N. humifusa x N. aticoana	H28 x A13	0
		Hu9-4 x A2	2_s
		Hu9-4 x A3	10_s
		Hu9-4 x A13	12_s
	N. humifusa x N. plicata	H28 x P11	0
		Hu1-2 x P11	7_s
		Hu1-2 x P5	8_s
		Hu9-4 x P5	1_f, 7_s
	N. plicata x N. humifusa	P11 x Hu9-4	0
	N. plicata x N. aticoana	P11 x A3	1_s

[z]Parentage = University of New Hampshire accessions codes of parent plants used in each hybridization.
[y]Germ. = Seedlings germinated out of fifteen mericarps sown per hybridization per storage treatment. Number seedlings germinated from fresh mericarps identified with subscript *f*, number of seedlings germinated from stored mericarps identified with subscript *s*.

APPENDIX B. Detail of fruit set, seed counts, and germination in interspecific hybridizations of *Nolana*. Seed counts determined by x-ray analysis of intact mericarps. Crosses refer to UNH accession codes of parent plants. All interspecific crosses resulting in fruit set are presented. X-ray and/or germination data are not available for all crosses.

Hybrid family[z]	Fruits developed[y]	Seeds per mericarp Mean[x]	Seeds per mericarp Range[w]	Mericarps sown	Seedlings germinated
N. adansonii x *N. aticoana*	**10**	**0.2**	**0-2**	**115**	**0**
Ad4-1 x A3	1	------	------	9	0
Ad4-14 x A2	3	0.1	0-1	36	0
Ad4-14 x A3	3	0.3	0-2	36	0
Ad4-14 x A13	3	0.1	0-1	34	0
N. adansonii x *N. plicata*	**19**	**0.2**	**0-2**	**209**	**0**
Ad2-2 x P7	1	0.1	0-1	10	0
Ad2-2 x P11	1	0.1	0-1	9	0
Ad4-1 x P5	1	------	------	1	0
Ad4-1 x P7	2	------	------	20	0
Ad4-1 x P11	1	------	------	12	0
Ad4-14 x P5	4	0.2	0-2	48	0
Ad4-14 x P7	4	0.3	0-2	51	0
Ad4-14 x P11	5	0.3	0-2	58	0
N. aticoana x *N. adansonii*	**11**	**1.7**	**0-4**	**60**	**19**
A13 x Ad2-2	5	2.0	1-3	25	9
A13 x Ad4-1	4	1.7	0-4	20	8
A13 x Ad4-14	2	1.4	0-3	15	2
N. aticoana x *N. humifusa*	**5**	**2.2**	**0-6**	**25**	**4**
A2 x Hu9-4	1	3.2	2-4	5	3
A3 x Hu9-4	2	1.4	0-3	10	1
A13 x H28	1	3.2	1-6	5	0
A13 x Hu9-4	1	2.6	1-4	5	0
N. aticoana x *N. laxa*	**18**	**2.5**	**0-5**	**93**	**0**
A2 x La1-2	3	2.6	1-5	14	0
A2 x La1-4	2	1.9	0-3	9	0
A2 x La3-1	5	2.1	0-4	24	0
A13 x La1-4	5	2.9	0-5	26	0
A13 x La3-1	3	2.5	1-5	20	0
N. aticoana x *N. plicata*	**37**	**3.4**	**0-10**	**111**	**52**
A2 x P7	5	5.1	4-10	23	21
A2 x P11	5	------	------	------	------
A3 x P5	4	2.9	0-5	20	7
A3 x P7	5	1.9	0-6	0	------
A3 x P11	5	2.8	1-7	23	3
A13 x P5	3	------	------	------	------
A13 x P7	5	3.7	2-5	15	8
A13 x P11	5	3.1	1-6	30	13
N. elegans x *N. aticoana*	**17**	**0.5**	**0-2**	**109**	**34**
Ele2 x A2	2	0.5	0-1	21	11
Ele2 x A3	2	------	------	------	------
051-3 x A2	4	0.9	0-2	15	8
051-3 x A3	3	0.8	0-1	5	1
051-3 x A13	2	0.3	0-2	9	6

APPENDIX B continued. Detail of fruit set, seed counts, and germination in interspecific hybridizations of *Nolana*.

Hybrid family[z]	Fruits developed[y]	Seeds per mericarp Mean[x]	Seeds per mericarp Range[w]	Mericarps sown	Seedlings germinated
051-5 x A2	2	0.1	0-1	53	5
051-5 x A3	2	0.5	0-2	6	3
N. elegans x *N. ivaniana*	**3**	**0.3**	**0-1**	**53**	**9**
051-5 x Iv2-1	3	0.3	0-1	53	9
N. elegans x *N. laxa*	**18**	**0.1**	**0-1**	**105**	**1**
051-3 x La1-2	3	------	------	38	0
051-3 x La3-1	5	------	------	35	0
051-5 x La1-2	3	------	------	------	------
051-5 x La1-4	3	------	------	------	------
051-5 x La3-1	4	0.1	0-1	32	1
N. elegans x *N. plicata*	**13**	**0.6**	**0-3**	**42**	**15**
Ele2 x P5	1	------	------	1	0
Ele2 x P7	1	------	------	1	0
Ele2 x P11	2	------	------	------	------
051-3 x P7	3	0.4	0-1	14	6
051-3 x P11	2	0.6	0-3	13	4
051-5 x P7	3	0.8	0-1	12	5
051-5 x P11	1	1.0	1	1	0
N. elegans x *N. rupicola*	**36**	**1.8**	**0-8**	**478**	**106**
Ele2 x Rup3	2	1.4	0-4	70	47
Ele2 x Rup1	1	------	------	10	5
Ele2 x Rup2	5	------	------	2	0
051-3 x Rup1	5	2.5	0-6	45	12
051-3 x Rup2	4	2.3	0-6	33	5
051-3 x Rup3	5	3.2	0-8	28	0
051-5 x Rup1	5	0.9	0-3	94	1
051-5 x Rup2	5	1.2	0-3	84	3
051-5 x Rup3	4	0.9	0-3	112	33
N. humifusa x *N. adansonii*	**14**	**1.0**	**0-5**	**64**	**4**
H28 x Ad2-2	5	0.9	0-5	22	0
H28 x Ad4-1	5	1.2	0-3	24	4
H28 x Ad4-11	4	0.8	0-3	18	0
N. humifusa x *N. aticoana*	**43**	**2.2**	**0-6**	**98**	**17**
H28 x A2	5	0.9	0-2	25	0
H28 x A3	5	1.1	0-4	25	0
H28 x A13	5	0.2	0-1	0	------
Hu1-2 x A2	5	3.4	1-6	25	11
Hu1-2 x A3	5	3.0	1-6	0	------
Hu1-2 x A13	5	2.8	1-5	23	6
Hu9-4 x A2	4	3.8	3-4	0	------
Hu9-4 x A3	5	------	------	------	------
Hu9-4 x A13	4	------	------	------	------
N. humifusa x *N. elegans*	**3**	**1.0**	**0-2**	**15**	**0**
H28 x Ele2	1	0.4	0-1	5	0
H28 x 051-3	2	1.3	0-2	10	0

APPENDIX B continued. Detail of fruit set, seed counts, and germination in interspecific hybridizations of *Nolana*.

Hybrid family[z]	Fruits developed[y]	Seeds per mericarp Mean[x]	Seeds per mericarp Range[w]	Mericarps sown	Seedlings germinated
N. humifusa x *N. ivaniana*	**35**	**2.0**	**0-9**	**220**	**3**
H28 x Iv2-1	5	1.4	0-4	35	0
H28 x Iv2-3	5	1.2	0-3	24	0
H28 x Iv2-5	5	1.5	0-4	34	0
Hu1-2 x Iv2-1	2	0.7	0-2	10	0
Hu1-2 x Iv2-3	5	2.2	0-4	25	0
Hu1-2 x Iv2-5	5	2.4	0-5	45	0
Hu9-4 x Iv2-1	5	3.3	0-9	32	0
Hu9-4 x Iv2-3	3	2.8	1-5	15	3
N. humifusa x *N. laxa*	**38**	**2.6**	**0-5**	**221**	**1**
H28 x La1-2	5	1.7	0-4	23	0
H28 x La1-4	5	1.5	0-4	24	1
H28 x La3-1	5	1.2	0-4	30	0
Hu1-2 x La1-2	2	2.7	1-5	24	0
Hu1-2 x La1-4	4	3.2	0-5	29	0
Hu1-2 x La3-1	5	2.9	2-4	31	0
Hu9-4 x La1-2	2	3.7	3-5	10	0
Hu9-4 x La1-4	5	3.9	2-5	25	0
Hu9-4 x La3-1	5	3.7	0-5	25	0
N. humifusa x *N. plicata*	**39**	**2.6**	**0-8**	**110**	**54**
H28 x P5	4	1.1	0-3	14	1
H28 x P7	5	1.9	0-5	33	2
H28 x P11	5	0.4	0-1	0	------
Hu1-2 x P5	5	------	------	------	------
Hu1-2 x P7	5	2.5	1-4	24	7
Hu1-2 x P11	5	------	------	------	------
Hu9-4 x P5	5	3.7	2-5	0	------
Hu9-4 x P7	3	4.3	2-8	29	30
Hu9-4 x P11	2	3.8	3-5	10	14
N. humifusa x *N. rupicola*	**14**	**1.1**	**0-9**	**76**	**0**
H28 x Rup1	5	1.8	0-9	33	0
H28 x Rup2	4	1.0	0-3	19	0
H28 x Rup3	5	0.7	0-3	24	0
N. ivaniana x *N. aticoana*	**8**	**2.2**	**0-5**	**42**	**8**
Iv2-1 x A2	4	2.1	0-5	20	3
Iv2-1 x A3	1	1.6	0-4	7	0
Iv2-1 x A13	3	2.5	0-5	15	5
N. ivaniana x *N. elegans*	**4**	**3.7**	**0-6**	**20**	**0**
Iv2-1 x 051-3	4	3.7	0-6	20	0
N. ivaniana x *N. laxa*	**34**	**3.1**	**0-8**	**204**	**4**
Iv2-1 x La1-2	3	3.1	0-6	13	1
Iv2-1 x La1-4	2	4.3	1-7	10	0
Iv2-1 x La3-1	3	2.4	0-6	14	0
Iv2-3 x La1-2	4	5.1	3-8	19	0
Iv2-3 x La1-4	5	4.8	1-8	35	0
Iv2-3 x La3-1	2	3.5	0-7	32	0

APPENDIX B continued. Detail of fruit set, seed counts, and germination in interspecific hybridizations of *Nolana*.

Hybrid family[z]	Fruits developed[y]	Seeds per mericarp Mean[x]	Seeds per mericarp Range[w]	Mericarps sown	Seedlings germinated
Iv2-5 x La1-2	5	2.0			0
Iv2-5 x La1-4	5	2.5	1-7	25	0
Iv2-5 x La3-1	5	1.8	0-5	36	3
N. ivaniana x *N. plicata*	**19**	**2.8**	**0-7**	**104**	**28**
Iv2-1 x P5	5	4.8	2-6	25	3
Iv2-1 x P7	3	3.1	0-7	25	6
Iv2-3 x P5	5	3.8	0-7	20	18
Iv2-5 x P5	5	1.0	0-4	29	1
Iv2-5 x P11	1	2.1	1-4	5	0
N. ivaniana x *N. rupicola*	**19**	**4.0**	**1-7**	**94**	**18**
Iv2-1 x Rup1	5	4.8	1-7	25	0
Iv2-1 x Rup2	2	3.7	1-6	10	0
Iv2-1 x Rup3	3	4.0	1-7	15	2
Iv2-3 x Rup2	4	3.8	1-6	19	5
Iv2-3 x Rup3	5	4.0	1-7	25	11
N. laxa x N. aticoana	**9**	------	------	**27**	**3**
La1-4 x A2	5	------	------	17	1
La1-4 x A3	1	------	------	------	------
La1-3 x A13	3	------	------	10	2
N. laxa x N. humifusa	**15**	------	------	**37**	**4**
La1-2 x Hu9-4	5	------	------	12	4
La1-4 x H28	1	------	------	------	------
La1-4 x Hu1-2	1	------	------	4	0
La1-4 x Hu9-4	4	------	------	14	0
La3-1 x Hu9-4	4	------	------	7	0
N. laxa x N. ivaniana	**34**	------	------	**92**	**4**
La1-2 x Iv2-1	5	------	------	17	0
La1-2 x Iv2-3	5	------	------	19	0
La1-2 x Iv2-5	5	------	------	11	0
La1-4 x Iv2-1	3	------	------	8	0
La1-4 x Iv2-3	4	------	------	15	3
La1-4 x Iv2-5	4	------	------	4	1
La3-1 x Iv2-3	4	------	------	18	0
La3-1 x Iv2-5	4	------	------	------	------
N. laxa x N. plicata	**32**	------	------	**89**	**21**
La1-2 x P7	2	------	------	9	7
La1-2 x P11	5	------	------	12	5
La1-4 x P5	3	------	------	6	0
La1-4 x P7	5	------	------	16	5
La1-4 x P11	5	------	------	13	0
La3-1 x P5	3	------	------	12	0
La3-1 x P7	5	------	------	19	4
La3-1 x P11	4	------	------	2	0
N. laxa x N. rupicola	**6**	------	------	**19**	**0**
La1-4 x Rup1	1	------	------	------	------
La1-4 x Rup2	3	------	------	12	0

APPENDIX B continued. Detail of fruit set, seed counts, and germination in interspecific hybridizations of *Nolana*.

Hybrid family[z]	Fruits developed[y]	Seeds per mericarp Mean[x]	Seeds per mericarp Range[w]	Mericarps sown	Seedlings germinated
La1-4 x Rup3	2	------	------	7	0
N. plicata x *N. aticoana*	**39**	**3.6**	**0-11**	**179**	**8**
P5 x A2	5	7.0	4-11	16	1
P5 x A3	3	4.3	0-9	29	1
P5 x A13	4	5.3	3-8	26	2
P7 x A2	5	2.5	0-4	4	0
P7 x A3	4	2.5	1-5	20	0
P7 x A13	5	2.2	1-4	35	0
P11 x A2	5	4.5	2-10	23	1
P11 x A3	4	3.1	0-6	26	3
P11 x A13	4	4.2	2-10	0	------
N. plicata x *N. humifusa*	**4**	**2.1**	**0-5**	**25**	**0**
P7 x Hu9-4	1	0.9	0-2	10	0
P11 x Hu9-4	3	2.8	1-5	15	0
N. plicata x *N. laxa*	**4**	**0**	**0**	**13**	**0**
P5 x La1-2	1	0	0	4	0
P5 x La1-4	2	0	0	6	0
P5 x La3-1	1	0	0	3	0
N. rupicola x *N. elegans*	**35**	**1.5**	**0-7**	**256**	**0**
Rup1 x Ele2	5	1.0	1	9	0
Rup1 x 051-3	3	1.9	0-7	0	------
Rup1 x 051-5	5	2.0	0-7	42	0
Rup2 x Ele2	5	1.3	0-4	48	0
Rup2 x 051-3	5	1.3	0-4	70	0
Rup2 x 051-5	1	0.7	0-4	13	0
Rup3 x Ele2	4	1.3	1-2	22	0
Rup3 x 051-3	5	1.6	0-5	47	0
Rup3 x 051-5	2	1.4	1-2	5	0

[z]Mericarps produced through artificial interspecific hybridization at the University of New Hampshire 2005-2006. Crosses refer to UNH accession codes of parent plants.

[y]Fruits developed out of five pollinations per accession cross or forty-five pollinations per species cross.

[x]Seeds per mericarp estimates made by x-ray analysis of a random set of mericarps from each species or hybrid family.

[w]Range = minimum and maximum number of seeds observed in individual mericarps.

APPENDIX C. Embryo Rescue Medium

(gm/ 2 liters)
1.35 $(NH_4)_2SO_4$
1.30 KNO_3
2.73 KCl
0.50 $MgSO_4 \cdot 7H_2O$
0.30 $CaCl_2 \cdot 2H_2O$
0.30 $NaH_2PO_4 \cdot H_2O$
30.0 sucrose
150 baby food banana
20.0 MS Micro (ml)
20.0 Fe-EDTA (ml)
5.00 gelrite
pH= 5.2

Fe/EDTA Stock (gm/L)
2.784 $FeSO_4 \cdot 7H_2O$
3.724 Na_2EDTA

MS Micro Stock (gm/L)
0.042 KI
0.310 H_3BO_3
1.120 $MnSO_4 \cdot 2H_2O$
0.430 $ZnSO_4 \cdot 7H_2O$
0.013 Na_2MoO_4

*Modified from Hinnen et al., 1989. Sci. Hort. 41:105.